Single-cell RNA profiling of *Plasmodium vivax* liver stages reveals parasite- and host-specific transcriptomic signatures and drug targets

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Running Head: Single-cell RNA profiling of *P. vivax* liver stages

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ABSTRACT

The resilience of *Plasmodium vivax*, the most widely distributed malaria-causing parasite in humans worldwide, is attributed to its ability to produce dormant liver forms known as hypnozoites, which can activate weeks, months, or even years after an initial mosquito bite. The factors underlying hypnozoite formation and activation are poorly understood, as is the parasite's influence on the host hepatocyte. Here, we shed light on transcriptome-wide signatures of both the parasite and the infected host cell by sequencing over 1,000 *P. vivax* liver forms at single-cell resolution. We distinguish between replicating schizonts and hypnozoites at the transcriptional level, identifying key differences in transcripts encoding for RNA-binding proteins associated with cell fate. In infected hepatocytes, we show that genes associated with energy metabolism and antioxidant stress response were upregulated, and those involved in the host immune response downregulated, suggesting both schizonts and hypnozoites alter the host intracellular environment. The transcriptional markers in schizonts, hypnozoites, and infected hepatocytes revealed here pinpoint potential factors underlying dormancy and can inform drug targets against *P. vivax* liver-stage infection.

IMPORTANCE

In humans, malaria-causing *Plasmodium* parasites invade and replicate in the liver before mounting an infection in the blood. A few *Plasmodium* species, including *P. vivax*, can cause relapsing infections due to forms that persist in the liver known as hypnozoites. While interventions targeting the hypnozoite can prevent clinical malaria, little is known about their biology at the molecular level. Transcriptome-wide assessment of individual hypnozoites has not been accessible due to the technical challenges associated with liver stage infections. To overcome technical limitations, we performed single-cell transcriptional profiling of liver stages generated using an *in vitro* infection platform. In elucidating the transcriptional signatures of *P. vivax* liver forms, as well as hepatocytes in response to infection, our data provide new insights into these host-parasite interactions. An improved understanding of these interactions will allow for novel drug or vaccine interventions targeting either parasite- or hepatocyte-specific metabolic pathways essential for successful liver stage development and disease progression.

INTRODUCTION

Malaria is a significant global health burden, with an estimated 421 million cases and 627,000 deaths per year (World Health Organization, 2021). At least five *Plasmodium* species are known to infect humans, with *P. vivax* being responsible for the majority of cases outside Africa (World Health Organization, 2021). All *Plasmodium* parasites that infect humans have an obligatory developmental stage in the liver wherein the parasite undergoes asexual reproduction known as schizogony within a

hepatocyte before releasing thousands of merozoites into the blood. In *P. vivax*, a proportion of parasites known as hypnozoites forgo immediate division in hepatocytes, and persist in the liver for weeks, months, or years before activating to initiate schizogony resulting in a relapse blood infection (Adams and Mueller, 2017; Krotoski et al., 1982). Hypnozoites are thought to cause up to 90% of cases in certain geographical regions (Adekunle et al., 2015; Commons et al., 2020; Robinson et al., 2015), and mathematical models predict that eliminating *P. vivax* malaria will be difficult without interventions that target hypnozoite reservoirs (White et al., 2018).

The factors influencing the biogenesis, persistence, and activation of hypnozoites are poorly understood. Studies have found that hypnozoites in temperate regions have long latencies and that tropical ones have short latencies, suggesting a correlation between geographical region and relapse patterns ((Battle et al., 2014; Huldén et al., 2008; Krotoski et al., 1986; White, 2011). These findings suggest that the developmental trajectory is genetically encoded, and support the hypothesis that developmental fate is somehow pre-programmed in the sporozoite (Lysenko et al., 1977; Ungureanu et al., 1976); but other contributing factors from the host could play a role in determining whether the sporozoite becomes a schizont or hypnozoite (Schäfer et al., 2021).

Our understanding of *P. vivax* liver stage biology at the cellular and molecular level is in its nascency for two overarching reasons. For a long time, protocols to cryopreserve and continuously maintain erythrocytic stage parasites *in vitro* were lacking, which necessitated the collection of gametocyte-infected blood from human volunteers in *P. vivax* malaria-endemic regions to generate the sporozoites required to initiate an infection. New laboratory models have recently been developed which can partially alleviate the need to obtain gametocytes from human infected blood (Mikolajczak et al., 2015; Schäfer et al., 2020). In addition to the sporozoite limitation, upon infecting the liver the high ratio of noninfected versus infected hepatocytes has historically made it difficult to isolate and study these forms in sufficient numbers. Robust *in vitro* pre-erythrocytic platforms capable of long-term culture of sufficient numbers of *P. vivax* liver stages have only recently been developed (Maher et al., 2021; Roth et al., 2018). Much of our knowledge of hypnozoite biology at the molecular level remains limited to studies using *P. cynomolgi*—a parasite capable of causing relapsing infections non-human primates, and for which molecular tools are available (Chua et al., 2019; Cubi et al., 2017; Voorberg-van der Wel et al., 2017, 2020). However, it is unknown whether mechanisms regulating hypnozoite formation and dormancy are consistent across different relapsing malaria parasites.

Transcriptome-wide profiling of *P. vivax* liver stages has previously been performed using a bulk RNA sequencing strategy (Gural et al., 2018). While informative, the data only provide insights into average transcript levels derived from either mixed (schizont and hypnozoite) or hypnozoite-only populations, masking any transcriptional variation that might exist between individual parasites. Second,

the parasite's potential impact on the hepatocyte is not considered, barring analysis of host-pathogen interactions. Nevertheless, these shortcomings highlight a need to better understand differences between individual parasites, as well as how the parasite alters the hepatocyte. Advancements in these areas could help elucidate parasite- and host- factors important for the parasite's liver stage development.

Single-cell RNA-sequencing (scRNA-seq) methods offer new ways of studying cell-to-cell variation (Aldridge and Teichmann, 2020), and have already allowed for novel insights into host-pathogen interactions in various disease contexts (Penaranda and Hung, 2019). In *Plasmodium* spp., scRNA-seq analyses have so far revealed that individual parasite forms exist on a spectrum of transcriptomic states (Bogale et al., 2021; Howick et al., 2019; Poran et al., 2017; Real et al., 2021; Reid et al., 2018; Ruberto et al., 2021a), and applying them to study the biology of *P. vivax* is a burgeoning area of research (Mancio-Silva et al., 2021; Ruberto et al., 2021b; Sà et al., 2020). Droplet-based techniques, such as 10x Genomics (Zheng et al., 2017), which use microfluidics, allow for the sequencing of tens to hundreds of thousands of cells, increasing the chances of observing rare cell populations (e.g., hepatocytes infected with *P. vivax*). We therefore presumed that applying single-cell sequencing to *P. vivax* liver forms derived using an *in vitro* platform would prove a fruitful avenue of inquiry into the parasite's biology cellular and molecular levels.

Using scRNA-seq, we characterize transcriptomic signatures of parasite and host in an *in vitro* *P. vivax* liver stage model. We find differences in gene expression between replicating schizonts and hypnozoites, and reveal variation, previously unobservable by bulk sequencing approaches, between individual hypnozoites. In infected hepatocytes, we find that genes associated with energy metabolism and antioxidant stress response are upregulated, and those involved in the host immune response downregulated. Various host genes associated with these processes are found to be exclusively upregulated during hypnozoite infection. Our study elucidates the transcriptional signatures among infected hepatocytes and presents potential mechanisms *P. vivax* can use to either exploit its host for rapid proliferation or to quietly persist, remaining undetected by the host immune system.

RESULTS

Strategy used to study *P. vivax* liver stage biology

Primary human hepatocytes were cultured and infected with *P. vivax* sporozoites on a 384-well plate platform (Maher et al., 2021; Roth et al., 2018). **Figure 1A** outlines the workflow for the experiment. Following infection with sporozoites, cultures were assessed at two endpoints: one containing both schizonts and hypnozoites at day 5 post-infection, and the other containing solely hypnozoites at day 9 post-infection. Schizonts were eliminated from day 9 post-infection cultures by adding the

phosphatidylinositol 4-kinase (PI4K) inhibitor MMV390048 (1 μ M) to the culture media on days 5, 6 and 7 post-infection (Gural et al., 2018; Paquet et al., 2017). For each collection time point, we simultaneously initiated two sets of hepatocyte cultures: one infected with sporozoites for quantification of immunofluorescent-labeled parasites by high-content imaging (HCI), and one infected with sporozoites for transcriptional profiling using a high-throughput, droplet-based scRNA-seq workflow (Zheng et al., 2017). As controls, a third set of naïve cultures were left uninfected and processed for scRNA-seq alongside cultures at days 5 and day 9 post-infection. At each collection point, a target of approximately 10,000 individual hepatocytes were partitioned into droplets prior to the generation of RNA libraries. Each droplet contained primers with a unique cellular barcode that was ligated to transcripts during the reverse transcription step, allowing for the identification of each transcript's cellular origin post-sequencing. After sequencing the single-cell libraries, we mapped the resulting reads onto the human (Ensembl, v103) and *P. vivax* PVP01 (PlasmoDB, v51) transcriptomes. We combined the transcript information with the cellular barcodes to assess the number of transcripts in each cell, and to determine whether a hepatocyte contained parasite material.

As expected, we detected hepatocytes infected with both forms of the parasite at 5 days post-infection, and only hypnozoites at 9 days post-infection following exposure to MMV390048 (**Fig. 1B and Fig. S1A**). **Figure 1C** displays the distribution of individual parasite growth areas, number of parasites detected, and parasitemia levels at each endpoint. Across replicates, the parasitemia ranged from 5.50-6.93% at 5 days post-infection and 1.76-2.77% at 9 days post-infection. Together, the high infection rates coupled with the high-throughput capacity of 10x Genomics' single-cell isolation and sequencing workflow allowed for sufficient material to be generated for successful profiling of both parasite and host transcriptomes.

***P. vivax* schizonts and hypnozoites are distinguishable at single-cell resolution**

We first analyzed the reads mapping to the *P. vivax* transcriptome to identify schizonts and hypnozoites, as well as to assess the extent to which these forms differed at single-cell resolution. In total, we characterized 1,438 parasite transcriptomes across two independent experimental replicates (**Fig. S1B**). The number of parasite transcriptomes profiled corroborated the parasitemia levels quantified using high-content imaging (**Table S1**). Low dimensional representation of the data revealed two populations of parasites in samples profiled at 5 days post-infection (**Fig. 2A, left panel**). In contrast, and as expected, samples profiled at 9 days post-infection contained one population (**Fig. 2A, right panel**). Given that the population of cells profiled at day 9 post-infection, from which schizonts were chemically removed, overlapped with one of the two cell populations profiled at 5 days post-infection, we deduced that the non-overlapping population of cells encoded for schizonts. Consistent with our reasoning, an unsupervised clustering algorithm (Traag et al., 2019) unbiasedly assigned the cells to one of the two

clusters (**Fig. 2B and Fig. S1C**). As expected, number of transcripts detected, unique molecular identifier (UMI) counts (a readout for absolute transcript abundances (Kivioja et al., 2011), and the transcript levels of the early liver stage development marker liver stage-specific protein, LISP2 (PVP01_0304700) (Gupta et al., 2019), all displayed greater quantities in the schizont cluster (C2) relative to the hypnozoite cluster (C1) (**Fig. 1C**). Together, these findings show that the transcriptomes of *P. vivax* schizonts and hypnozoites can be distinguished using our experimental workflow.

Hypnozoites exhibit distinct transcriptional signatures compared to schizonts

We next performed differential gene expression analysis to identify transcriptome-wide differences distinguishing schizonts and hypnozoites. Using stringent inclusion cutoffs (adjusted p value < 0.01 & absolute (average log₂ fold change [FC]) > 0.5), we found significant differences in ~18% (836/4722) of all transcripts detected (**Fig. S2A; Data Set S1A**). Relative to hypnozoites, we found an increase in the expression levels of 368 genes in schizonts. As expected, we observed a significant increase of LISP2 in schizonts (average log₂ FC: 2.32) compared to hypnozoites. Shortly after invading the hepatocyte, non-relapsing *Plasmodium* spp. liver forms undergo immediate replication. Studies in rodent malaria models have shown that these forms are metabolically active and that various genes involved in energy metabolism are upregulated (Caldelari et al., 2019; Tarun et al., 2008; Toro-Moreno et al., 2020). Consistent with previous data, we found an increased transcription associated with glycolysis (PVP01_0816000, PVP01_1229700, PVP01_1244000) and the TCA cycle (PVP01_0106100, PVP01_1332400) in schizonts (**Fig. S2B; Data Set S1A**). More broadly, we found increased transcription of various metabolic processes, as well as motility, DNA-, RNA-, and protein-processing (**Fig. S2C; Data Set S1A**). Transcripts encoding for various proteasome subunits displayed greater transcription in schizonts (**Fig. S3A; Data Set S1A**). To further show the importance of this protein complex, we tested a known proteasome inhibitor, Carfilzomib (Kortuem and Stewart, 2013), in our standard 12-day *P. vivax* small molecule screening assay (Maher et al., 2021), and found it effectively killed schizonts (IC₅₀ 288nM) and hypnozoites (IC₅₀ 511nM) with some selectivity over host hepatocytes (IC₅₀ 3.32 μM) (**Figs. S3B and S3D**).

The detection of transcripts associated with energy metabolism in hypnozoites, albeit at a lower level relative to schizonts, suggested that these forms are also metabolically active (**Fig. S2B**). Relative to replicating schizonts, we found an increase in gene expression of 468 transcripts in hypnozoites (**Data Set S1A**). As represented in **Figure 3A**, genes with greater expression in hypnozoites encoded for a diverse set of ontologies—including proteases, membrane proteins, transcription factors, and DNA-/RNA-regulating proteins. Genes encoding for proteases vivapain-1, vivapain-2 (PVP01_1248900, PVP01_0916200), and plasmepsin IV (PVP01_1340900) displayed the greatest differential expression in these forms compared to schizonts (**Figs. 3A and 3B; Data Set S1A**). While limited research has

reported the functionality of these gene products in *P. vivax* (Byoung-Kuk et al., 2004), work in other *Plasmodium* spp. has revealed that they localize to the food vacuole and play an important role in hemoglobin catabolism during the blood stages of the parasite's life cycle (Dame et al., 2003; Drew et al., 2008).

We also identified genes with greater expression associated with RNA regulatory mechanisms. Notably, we found greater expression of CCR4 (PVP01_1429000), a transcript encoding for a protein shown to have mRNA deadenylase activity and play a role in mRNA turnover (Tucker et al., 2001) (**Data Set S1A**). In *Plasmodium* spp., it has been described as important in regulating the expression of genes associated with invasion and egress in blood stages (Balu et al., 2011). RNA-binding proteins (RBPs) also govern gene expression at the post-transcriptional level by transiently storing transcripts until later processing (Hentze et al., 2018). Pumilio domain (PUF) proteins are a major family of RBPs that regulate cellular fating through translational repression in eukaryotes. We found that the transcript encoding for PUF1 (PVP01_1015200) was detected almost exclusively in hypnozoites (**Figs. 3A and 3B; Data Set S1A**). Furthermore, we found greater expression of 7-Helix-1 protein (PVP01_1008400) (**Figs. 3A and 3B; Data Set S1A**)—a stress granule component that interacts with the RNA-binding protein PUF2 and serves to be crucial for protein synthesis in *P. falciparum* (Miao et al., 2010). Numerous studies have shown that *Plasmodium* spp. use various post-transcriptional regulatory mechanisms to coordinate stage transitions and development (Baumgarten et al., 2019; Bunnik et al., 2016; Foth et al., 2011; le Roch et al., 2004; Tarun et al., 2008; Vembar et al., 2016). Our data corroborate these findings and suggest that similar strategies may be used in *P. vivax* to regulate liver stage development. Moreover, these data suggest that RBP-mediated cellular fating and translational repression may be involved in regulating hypnozoite dormancy.

Given the potential role in RBP-mediated cellular fating and translational repression governing latency in hypnozoites, we performed further analyses focused on RBP-mediated regulation. To this end, we scanned the *P. vivax* genome searching for genes encoding for cellular fating RBPs based on ortholog groups (OGs) of known human cellular fating RBPs (348 RBPs; 227 OGs), identifying 89 OGs (99 RBPs) in *P. vivax*. We detected 48 genes encoding for cellular fating RBPs with greater expression in schizonts (**Fig. 3C, left panel; Data Set S1B**). Notably, we identified eukaryotic translational initiation factors (PVP01_1238100, PVP01_0531200, PVP01_1431600, PVP01_0807600, PVP01_1005600, PVP01_0519700, PVP01_1329200, PVP01_0825000, PVP01_1303500), and splicing factors (PVP01_0716000, PVP01_0521000, PVP01_0804100, PVP01_1111800, PVP01_0607600, PVP01_1012200) suggesting active translational events. Gene Ontology (GO) term analysis revealed that these transcripts were associated with translation initiation (GO:0005852; GO:0033290; GO:0016282) (adjusted p value < 0.05, **Fig. 3C; Data Set S1C**).

In hypnozoites, we detected 51 genes encoding for cellular fating RBPs with increased expression relative to schizonts (**Fig. 3C, left panel; Data Set S1B**). These transcripts encode for prominent P body markers, namely DOZI/DDX6 (PVP01_0819400), CCR4-NOT complex proteins (PVP01_1429000, PVP01_1014400, PVP01_1331700, PVP01_1453400, PVP01_0929400), mRNA-decapping enzyme subunit 1 (PVP01_0617200), mRNA-decapping enzyme subunit 2 (PVP01_1409900), UPF1 (PVP01_0805200), UPF2 (PVP01_0724300), UPF3 (PVP01_1226700), PUF2 (PVP01_0526500), PABP (PVP01_1442500), Fibrillarin (PVP01_1341600) and trailer hitch homolog (PVP01_1269800) (**Data Set S1B**). Transcripts encoding for cellular fating RBPs in hypnozoite also represented GO terms corresponding to active biological condensate formations, such as the CCR4-NOT complex (GO:0030014; GO:0030015) and the P body (GO:0000932) (adjusted p value < 0.05, **Fig. 3C, right panel; Data Set S1C**). Together, these findings suggest that P body-mediated cell fating could be important in regulating hypnozoite persistence.

Transcriptomic heterogeneity exists amongst individual *P. vivax* hypnozoites

Having described the transcriptomic signatures distinguishing schizonts and hypnozoites, we next sought to assess the extent to which gene expression varied amongst hypnozoites. We thus parsed the data into only hypnozoite transcriptomes. In total, 1,147 transcriptomes were assessed (**Fig. 4A, left panel**). Low dimensional representation of the data revealed slight variation between samples collected at days 5 and 9 post-infection (**Fig. 4A, right panel**). After regrouping the data using the same unsupervised clustering algorithm described previously, we identified three clusters (**Fig. 4B, right panel**). The proportion of hypnozoites in each cluster varied across the day of collection: specifically, the proportion of hypnozoites in cluster 3 (C3) was greater in hypnozoites collected at day 9 compared to day 5 post-infection (**Fig. 4B, left panel; Fig. S4A**). Notably, we also found greater gene expression of LISP2 in C3 relative to the other two clusters (**Fig. 4C**).

We next identified markers that define the hypnozoites in each cluster using the FindAllMarkers function in Seurat (Stuart et al., 2019). We defined a marker as a transcript displaying greater than 0.5 FC (average log₂) compared to the other clusters. In total, we detected 421 markers (adjusted p value < 0.05) (**Fig. S4B; Data Set S2A**). Notable markers in C1 included transcripts associated with drug resistance (PVP01_1447300, PVP01_1259100, and PVP01_1010900), energy metabolism (PVP01_1435000 and PVP01_1122800), protein modification (PVP01_1248900 and PVP01_0916200), and RNA-binding proteins (PVP01_0939900 and PVP01_0819400); in C2 those associated with translation (PVP01_1255500 and PVP01_0822500); and in C3 those associated with translation (PVP01_0213700 and PVP01_0203400), fatty acid metabolism (PVP01_1143000 and PVP01_1022800), protein processing (PVP01_1308000 and PVP01_0927700) and ion transport (PVP01_1407500) (**Fig. 4D; Data Set S2A**).

We next explored the data in the context of cellular fating to elucidate potential patterns in RBP signatures. Among the hypnozoite markers found within our sub-clustering analysis, we identified 68 cellular fating RBPs, of which 54 RBPs were differentially expressed in at least in one of the three clusters (adjusted p value < 0.05) (**Fig. 4E, top; Data Set S2B**). Approximately 4% (2/54) of the differentially expressed genes in C3 encoded for cellular fating. In this cluster, the increased gene expression of eukaryotic translation initiation factor 4E (PVP01_0203400) further suggests active translation events may be occurring. C2 contained ~16% (9/54) of the cellular fating RBPs differentially expressed. In this cluster, we found RBPs which are important determinants of cell proliferation from a state of senescence such as eukaryotic translation initiation factor 5A (PVP01_1303500), polypyrimidine tract binding protein (PVP01_1142400), GTP-binding nuclear protein RAN/TC4 (PVP01_0918300), transformer-2 protein homolog beta (PVP01_0802200). C1 contained the majority of the differentially expressed RBPs (~80%, 43/54). Within this cluster, we identified prominent P body markers with cellular fating ability, such as DOZI/DDX6 (PVP01_0819400), CCR4-NOT complex proteins (PVP01_1429000, PVP01_1014400, PVP01_1331700, PVP01_1453400), UPF1 (PVP01_0805200), UPF2 (PVP01_0724300), PABP (PVP01_1442500), Fibrillarin (PVP01_1341600) and trailer hitch homolog (PVP01_1269800). GO analysis revealed enrichment of cellular compartments associated to biological condensate formations, namely CCR4-NOT complex (GO:0030014), P granule (GO:0043186), and germplasm (GO:0060293) (**Fig. 4E; Data Set S2C**).

These findings, combined with the shift of hypnozoite proportions and increased transcript levels of LISP2 from C1 to C3, allowed us infer the phenotype of hypnozoites in these clusters. As these observations likely represent populations of hypnozoites in persisting and activating states, we defined clusters C1, C2, and C3 to represent persisting, transitioning, and activating hypnozoites, respectively. Together, these findings further our understanding of *P. vivax* hypnozoites through characterization of gene usage and putative regulatory mechanisms in activating and persister forms. Furthermore, they highlight a potential role of P body-mediated cellular fating governing persister hypnozoites.

Strategy used to assess hepatocytes infected with *P. vivax* parasites

After deciphering the identities of *P. vivax* transcriptomes representing schizonts and hypnozoites, we next sought to characterize the transcriptional signatures of hepatocytes infected with either of these forms. To this end, we integrated the data across all the conditions (**Figs. S5A and 5B**) and examined whether distinct sub-populations of infected hepatocytes existed. As highlighted by the low dimensional visualization of the data, we did not find a prominent pattern of infection (**Fig. 5**). Moreover, we detected parasites in hepatocytes with different metabolic states (MacParland et al., 2018), as represented by their transcriptional signatures associated with different hepatocyte zonation programs (**Figs. S5C and S5D**).

Identification of transcriptional signatures in *P. vivax*-infected hepatocytes

To identify host transcriptional signatures specific to infection status, we performed differential gene expression analysis, comparing infected to non-infected hepatocytes. Among the hepatocytes infected with schizonts, 106 genes displayed greater expression and 16 displayed decreased expression (**Data Set S3A**); while in hepatocytes infected with hypnozoites, 365 genes displayed greater expression and 41 genes displayed decreased expression (**Data Set S3B**) (adjusted p value < 0.05). As depicted in the volcano plots in **Fig. 6A**, the magnitude of change in expression in the infected versus non-infected cells (average log₂ fold change) was greater in hepatocytes containing schizonts compared to those containing hypnozoites.

To gain greater insights into the biological processes and pathways that characterize a schizont- or hypnozoite-infected hepatocyte, we performed enrichment analysis (Raudvere et al., 2019)—scoring 18,594 gene sets against the genes differentially expressed in each of our transcriptional comparisons. When considering both the schizont- and hypnozoite-infected hepatocyte populations, we detected a total of 254 enriched gene sets (adjusted p value < 0.05) (**Fig. 6B**; **Data Set S3C**). Approximately 18% (45/254) of these gene sets overlapped in hepatocytes infected with either schizonts or hypnozoites (**Fig. S5E**; **Data Set S3D**). These data indicate that while some overlap exists in the gene networks the parasite alters during infection, the majority of processes and pathways altered are specific to schizont versus hypnozoite infected hepatocytes.

Using these gene sets, we then constructed network enrichment maps (Merico et al., 2010) to provide a global overview of the prominent biological themes associated with each infection condition (**Figs. 6C and 6D**). We detected upregulation of genes associated with pathways related to energy metabolism (oxidative phosphorylation, mitochondrial function, and lipid catabolism) in schizont- and hypnozoite-infected hepatocytes. Analysis of the most differentially expressed genes associated with these pathways included *MT-ND2*, *CHCHD10*, and *AKR1B10* (**Data Sets S3A and S3B**). Corroborating our transcriptomic data, we found an increase in protein levels of AKR1B10 in infected versus non-infected hepatocytes (**Figs. S6A-D**). AKR1B10 has been implicated in cell survival through its regulation of lipid metabolism and elimination of carbonyls (Martin and Maser, 2009; Qu et al., 2021; Wang et al., 2009) and has primarily been described as a biomarker of cancer (Díez-Dacal et al., 2011; Fukumoto et al., 2005; Jin et al., 2006). More recently, it has been shown that its expression is positively correlated with non-alcoholic fatty liver disease (Govaere et al., 2020). Its role during *Plasmodium* spp. liver stage infection warrants further research. In schizonts, the upregulation of genes linked to fatty acid metabolism is consistent with observations in hepatocytes harboring developing forms from non-relapsing *Plasmodium* spp. (Albuquerque et al., 2009; Itoe et al., 2014). We now reveal that infection

with hypnozoites also elicits enrichment of transcripts associated with fatty acid metabolism (**Fig. 6D**), suggesting that these forms may be scavenging host resources to fuel their persistence.

Previous work assessing *Plasmodium* liver stage biology has revealed that the host is able to detect the parasite (Liehl and Mota, 2012) and that type I interferon plays an integral role in this response (Liehl et al., 2013; Miller et al., 2014). The decrease in transcripts associated with interferon signaling (REAC:R-HSA-913531, GO:0034340, GO:0034341, GO:0071357, GO:0060337) in hepatocytes infected with either schizonts or hypnozoites suggest that *P. vivax* manipulates the hepatocytes to evade detection (**Figs. 6C and 6D; Data Sets S3C and S3D**). In hepatocytes infected with schizonts, we found a decrease in expression of NFkB-regulated genes (*SOD2*, *TMEM45A*, *CCL20*, and *TPMI*) (**Data Set S3A**), consistent with findings that *Plasmodium* spp. release signals to block proinflammatory response (Singh et al., 2007). These findings, in addition to the decrease in the expression of *HLA-B*—a molecule involved in antigen presentation to T cells that can trigger their activation when ‘self’ peptides are not presented—and the neutrophil chemoattractant *CXCL8* suggest that replicating parasites alter hepatocyte signaling pathways to minimize recruitment of innate immune cells and avoid detection by T cells.

Similar to hepatocytes infected with schizonts, we found genes associated with interferon signaling downregulated in hepatocytes infected with hypnozoites, namely: *UBB*, *UBC*, *HSP90AB1*, *IFITM3*, *IFITM2*, and *HLA-A* (**Data Set S3B**). Furthermore, transcripts associated with the downregulation of class major histocompatibility complex (*CD81* and *B2M*) were also decreased in these hepatocytes (**Data Set S3B**). Together, these findings suggest that *P. vivax* infection of hepatocytes may suppress the secretion of various chemokines and proinflammatory cytokines to thwart the recruitment of inflammatory cells to the site of infection while simultaneously reducing the chances they will be detected if these cells are recruited by downregulating MHC- I.

Enrichment analysis linking the changes in gene expression in infected hepatocytes with transcriptional factors revealed distinct sets of putative transcriptional regulators. In total, we detected 35 transcription factors with potential roles in governing the gene expression changes in infected hepatocytes (adjusted p value < 0. 05, **Fig. 7; Data Set S3E**). In infected hepatocytes, we found enrichment of transcripts governed by NRF2 (**Fig. 7; Data Set S3E**). The regulation of gene expression through NRF2 in infected hepatocytes may serve to minimize reactive oxygen species in the cell (Hayes and Dinkova-Kostova, 2014). Thus, in addition to the decreased expression of genes associated with inflammatory response (**Data Set S3C**), NRF2-mediated regulation of antioxidant responsive genes may protect hepatocytes from *Plasmodium*-induced stress and thus serve as a complementary means of minimizing the host inflammatory response.

DISCUSSION

The infection of the liver by *P. vivax* constitutes an obligatory step in its life-cycle. *In vivo* studies have shown that within hepatocytes, *P. vivax* can exist in one of two phenotypically distinct forms: schizonts or hypnozoites (Cogswell, 1992; Mikolajczak et al., 2015). They have also been observed *ex vivo* by infection of hepatocyte monolayers (Gural et al., 2018; Hollingdale et al., 1985; Roth et al., 2018; Sattabongkot et al., 2006). A key problem hindering the study of *P. vivax* liver stage biology is the difficulty involved in obtaining a sufficient number of infected hepatocytes to make meaningful comparisons between them. We show that by coupling our *in vitro* liver stage model (Maher et al., 2021; Roth et al., 2018) with a high throughput single-cell capture technology (Klein et al., 2015; Macosko et al., 2015; Zheng et al., 2017) transcriptome-wide characterization of a large number of infected hepatocytes is feasible. Our approach therefore provides much needed insight into the molecular signatures of *P. vivax* hypnozoites and the host cells they infect.

First, we found clear transcriptional differences between schizonts and hypnozoites. Our transcriptome-wide assessment supports the notion that hypnozoites are metabolically active (Adams and Mueller, 2017), demonstrated by the transcription associated with glycolysis and fatty acid metabolism. Second, we found that hypnozoites exist in various transcriptomic states. We speculate that these differences might represent a spectrum of phenotypes, from persisting to activating. The differential expression of genes encoding for cellular fating RBPs supports the notion that these forms may use post-transcriptional mechanisms to control gene usage (Mueller et al., 2019). The biological significance of the enrichment of transcripts associated with translational repression, found exclusively in a subpopulation of hypnozoites, suggests that this mechanism inhibits activation, and warrants further investigation.

Second, we found distinct transcriptomic signatures of infected versus non-infected hepatocytes, and showed that signaling pathways associated with energy metabolism, antioxidant stress, and immune response are altered in infected hepatocytes. We found no clear pattern of infection, as both schizonts and hypnozoites are detected in hepatocytes with various metabolic signatures. The extent to which individual hepatocytes respond to *P. vivax* schizonts and hypnozoites has previously been unknown. A bulk RNA sequencing approach has been used to identify pathways altered in hepatocytes in response to *Plasmodium* infection (LaMonte et al., 2019); however, it is limited insofar as it averages gene expression from millions of cells, obscuring any parasite-specific effects on the infected cell. Targeted approaches have revealed that *P. vivax* liver forms recruit host factors to facilitate growth (LaMonte et al., 2019; Posfai et al., 2020), but how certain infected hepatocyte populations contribute to disease transmission, and how hepatocyte subpopulations support development, are intriguing topics for future studies (Vijayan et al., 2021). Our data identify potential host factors essential for *P. vivax*'s

successful liver stage development. Whether certain hepatocyte phenotypes influence the development, especially activation of hypnozoites into schizonts, requires further investigation.

Several observations suggest protein degradation may be important for the parasite's liver stages. Targeting this process may be an effective means of killing these forms. The ability of hepatocytes to uptake and break down hemoglobin (Bissell et al., 1972) supports that this protein substrate could be an essential amino acid source for the parasite. Our data suggest that several mechanisms may allow *P. vivax* to utilize the amino acids derived from this protein. In hypnozoites, the increased expression of genes encoding for proteases, such as vivapains, supports the idea that these parasites digest host cytosolic proteins. The role of these proteins as hemoglobinsases have been defined extensively in *P. falciparum* blood stages (Drew et al., 2008; Rosenthal, 2011; Salas et al., 1995). A similar role has been described for *P. vivax* (Byoung-Kuk et al., 2004), but there is limited information about their significance in the liver stages. Vivapains are highly homologous to cathepsin protease L (TgCpl) in the related apicomplexan *Toxoplasma gondii*. In these parasites, TgCpl is localized to its plant-like vacuolar compartment—a lysosome-like organelle containing a variety of proteases (Parussini et al., 2010)—and one of its functions is to aid in the digestion of host-derived proteins (Dou et al., 2014). Genetic or chemical disruption of TgCpl negatively impacts the viability of bradyzoites, a persisting form of this parasite (di Cristina et al., 2017). Given these orthologous proteins' roles in *P. falciparum* and *T. gondii*, we speculate that the expression of vivapains may support the persistence of *P. vivax* hypnozoites.

Persistence of hypnozoites is contingent on the longevity of the hepatocyte in which it resides. The increased expression of genes associated with antioxidant stress (Scarpulla, 2002) is consistent with the notion that hypnozoite infection induces changes in the hepatocyte to promote cell survival, in which NRF-2 could be playing a regulatory role. We detected genes displaying greater expression in infected hepatocytes related to NYFB-regulation, a transcription factor important for cell proliferation, mitochondrial integrity, and cellular longevity (Tharyan et al., 2020). The increased expression of mitochondria-related genes in hepatocytes infected with schizonts may be required to meet the parasite's energy demands during replication, but the enrichment of mitochondria-related transcripts in cells infected with hypnozoites could also aid in ensuring longevity of the hepatocyte. In addition to these positive regulators of cell survival, we found negative regulation of transcripts through the decreased expression of genes associated with cell death (*UBB*, *UBC*, and *HSP90AB1*). Their expression is thought to be regulated by CEBPD, a transcription factor that has a role in governing cell death (Tsai et al., 2017; Wang et al., 2019).

Persistence of hypnozoites also requires evading host detection, but how they are able to do so has been elusive. Our analyses indicate that the parasite alters the host hepatocyte to evade detection by

immune cells. First, there is a decrease in expression of genes encoding for chemoattractants (*CCL20*, *CXCL8*, *IL32*) in infected hepatocytes, and an increase in expression of PTGR1, a negative regulator of the chemotactic factor leukotriene B4 (Yokomizo et al., 1993). These changes would result in decreased secretion of chemokines that recruit innate immune cells to the liver, thereby reducing recruitment of adaptive immune cells like CD8 T-cells that kill infected hepatocytes (Chakravarty et al., 2007). Interestingly, there was also a decrease in expression of MHC-1 molecule (*HLA-A* and *-B*). The presentation of parasite peptides to be detected by surveying CD8 T-cells would therefore be reduced (Neefjes et al., 2011), creating an additional layer of protection should innate immune cells begin to be recruited, for example upon lysis of the hepatocyte by schizonts. We propose that *P. vivax* infected hepatocytes are re-programmed to prevent recruitment of innate immune cells on the one hand, and to reduce the likelihood of their detection by the adaptive immune system on the other, all to enable progress through schizogony and for hypnozoites to persist undetected. Understanding the mechanisms leading up to this should be studied in the future, as their reversal may lead to improved clearance of hypnozoites, thus reducing relapses. Indeed, a vaccine would need to decrease relapses to have any impact on *P. vivax* transmission and disease (White et al., 2017). However, the decrease of MHC-I expression in infected hepatocytes may mean that standard focuses to generate CD8 and CD4 polyfunctional T-cells in the liver may not be effective in eliminating *P. vivax* or other relapsing parasites. Recent attempts to test anti-relapse vaccine candidates with *P. cynomolgi* did not prevent relapses despite higher inducing IFN γ producing cells, a common metric used as a correlate of protection (Kim et al., 2020), indicating that alternative strategies may be needed.

Our transcriptome-wide characterization of schizonts, hypnozoites, and the infected hepatocytes highlights transcripts altered during infection, and provides a framework for examining the role of infection dynamics of *P. vivax* liver stage infections. By examining two early liver stage time points at single-cell resolution, our study lays out proof of principle and a technical advance demonstrating the importance of *in vitro* models to shed light on the molecular basis of *P. vivax* liver stage development and infection.

CONCLUSIONS

The overall aim of our research was to elucidate the molecular signatures of *P. vivax* liver infection, a better understanding of which will aid in facilitating the development of new therapeutics to ultimately reduce transmission. We anticipate that interventions could involve parasite- and/or host- directed small molecule therapies, two approaches our group is actively pursuing. The comprehensive mapping of the transcriptional landscape of both the host and *P. vivax* described here provides an important framework for further investigation.

MATERIALS AND METHODS

Ethics statement

All research procedures were reviewed and approved by the Cambodian National Ethics Committee for Health Research (approval numbers: #113NECHR, #104NECHR and #089NECHR).

Blood samples, mosquitos, and infections

The complete protocol used for mosquito rearing an infection has been published (Maher et al., 2021). In brief, blood samples from symptomatic patients infected with *P. vivax* were collected at local health facilities in Monduliri province in Cambodia from March 2020 to October 2021. Following a *P. vivax* gametocyte-containing blood meal, *An. dirus* mosquitoes were maintained at ambient temperature on a 12:12 light: dark cycle and fed 10% sucrose + 0.5% PABA solution. *An. dirus* found positive for *P. vivax* oocysts at six-days post-feeding were transported to the Institut Pasteur Cambodia Insectary Facility in Phnom Penh, Cambodia where they were maintained under the same conditions described above.

Sporozoite isolation and primary human hepatocyte infections

The complete protocol used for seeding primary human hepatocytes, harvesting sporozoites, and infecting cultures has been published (Maher et al., 2021). In brief, *P. vivax* sporozoites were isolated from the salivary glands of female *An. dirus* mosquitoes 18 days after an infectious blood-meal. Primary human hepatocytes (Lot BGW, BioIVT) were seeded 2 days before infection with 30,000 sporozoites (replicate 1), or 15,200 sporozoites (replicate 2) per well. Media was replaced with fresh CP media (BioIVT) containing antibiotics (penicillin, streptomycin, and gentamycin) the day after infection and every 2-3 days thereafter. Hepatocytes containing solely hypnozoites at 9 days post-infection were treated with 1 μ M MMV390048 on days 5, 6, and 7 post-infection to kill replicating schizonts. At days 5 and 9 post-infection, cultures were processed for single-cell sorting (described in the following section) or immunofluorescence assay (IFA). For the latter, hepatocytes were fixed with 4% paraformaldehyde in 1X PBS. Fixed cultures were then stained overnight at 4°C with recombinant mouse anti-*P. vivax* UIS4 antibody (Schäfer et al., 2018) diluted 1:25,000 in a stain buffer (0.03% Triton X-100) and 1% (w/v) BSA in 1X PBS. The following day, cultures were washed three times with 1X PBS and then stained overnight at 4°C with 1:1,000 rabbit anti-mouse AlexaFluor™488-conjugated antibody diluted 1:1,000 in stain buffer. Cultures were washed three times with 1X PBS and counterstained with 1 mg/mL Hoechst 33342 (Thermo Fisher) to detect parasite and host DNA. High content imaging was performed on a Lionheart imaging system (Biotek). Quantification of the number

of nuclei and parasite liver forms was performed using Gen5 high content analysis software (Biotek). Confocal images were obtained using an ImageXpress Micro (Molecular Devices).

Single-cell partitioning, library preparation, and sequencing

Hepatocyte cultures infected with *P. vivax* were processed prior to- and post- PI4K treatment (5- and 9-days post-infection, respectively). Two replicate experiments, defined as an independent plating of hepatocytes infected with sporozoites from a unique field isolate, were performed several months apart. Additionally, and as a negative control, one replicate of hepatocyte cultures never exposed to *P. vivax* (naïve) were processed similarly to those that were infected. At each endpoint for each replicate, approximately 640,000 PHH hepatocytes (16 wells of a 384-well plate each having approximately 10,000 cells) were treated with trypsin (Corning 25-053-Cl) to facilitate their detachment from the plate. Once detached, trypsin was inactivated with 1:1 complete media and the cell suspension was collected into a 5mL protein-low bind centrifuge tube (Eppendorf). To remove residual trypsin, hepatocytes were washed via three sequential rounds of pelleting (50 x g, 3 min, 4°C) and resuspending in 1X PBS containing 0.1% BSA. Cells were then passed through a 35µm strainer (Falcon 352235) to minimize or remove hepatocyte clusters. Prior to partitioning cells on the Chromium controller (10x Genomics), hepatocytes were counted and the cell concentration was assessed using a hemocytometer. Approximately 10,000 hepatocytes were loaded on a Chromium Chip B (10x Genomics) as per manufacturer's recommendations. Chips containing hepatocyte suspensions, Gel Beads in Emulsion (GEMs), and reverse transcription reagents were loaded into the Chromium controller (10x Genomics) for single-cell partitioning and cell barcoding. Barcoded cDNA libraries were generated according to the Chromium Single Cell 3' gene expression protocol (version 3). In total, 6 cDNA libraries were generated (two replicates at 5 days post-infection, two replicates at 9 days post-infection, one uninfected control for day 5 cultures, and one uninfected control for day 9 cultures). Libraries were loaded on individual flow cell lanes and sequenced at a depth of 400 million reads using a HiSeq X Ten platform (Illumina) at Macrogen (Seoul, Korea).

Computational analysis of single-cell RNA sequencing data

Quality control checks on the raw sequencing data were performed using FASTQC (Andrews, 2010). A multi-species transcriptome index—containing the transcriptomes for *P. vivax* P01 (PlasmoDB.org, version 51) and *H. sapiens* GRCh8 (Ensembl version 103)—was created using the 'ref' function in kb-python (0.26.3) (Bray et al., 2016; Melsted et al., 2021). Next, reads pseudoaligned and counted using the 'count' function in kb-python with default parameters selected. The resulting unfiltered cell-gene count matrices were read into R (v 4.1.1) for further processing.

Filtering and normalization of scRNA-seq count matrices

R scripts containing all computational analyses will be available on a dedicated GitHub repository upon publication.

To distinguish between droplets containing cells and those only with ambient RNA, we used emptyDrops function (Lun et al., 2019) with the lower total UMI count threshold (at or below which all barcodes are assumed to correspond to empty droplets) set to 1,000. The droplets with cells (FDR < 0.001), more specifically, those containing barcoded transcripts derived from either human, parasite, or a combination of the two—were kept for downstream processing.

Assessment of *P. vivax* liver stage transcriptomes

For the analyses of *P. vivax*, we filtered out the transcripts derived from human, leaving only transcripts derived from the parasite quantifiable. For each sample, cells with less than 200 UMIs were removed. Next, we filtered out low detected genes, retaining transcripts with 10 or greater UMIs. Post cell and gene filtering, the data were normalized using Seurat (Stuart et al., 2019) ‘LogNormalize’ function with the default parameters selected.

Data integration and clustering

The data were integrated using Seurat’s SCTransform function (Hafemeister and Satija, 2019) with the following parameters indicated: variable.features.n = NULL, variable.features.rv.th = 1.3. Clustering was performed using Seurat’s SNN-Leiden algorithm (Traag et al., 2019). The full description of this analysis is found in the extended methods.

Differential gene expression analysis

To detect differentially expressed transcripts, the Seurat function FinalAllMarkers and FindMarkers were used. For differential gene expression testing using the FindMarkers function, we used the following parameters: logfc.threshold = 0.0, ident.1 = '1', ident.2 = '2', test.use = "wilcox", min.pct = 0, only.pos = F, assay = "RNA". For differential gene expression testing using the FindAllMarkers function, we used the following parameters: test.use = "wilcox", only.pos = T, assay = "RNA", log2FC = 0.0.

RBP analyses

RBPs with cell fating potential reported in humans (Decker and Parker; Gerstberger et al., 2014; Guallar and Wang, 2014; Voronina et al., 2011; Youn et al., 2019) were manually curated. The

orthologue's counterparts of these cell fating RBPs from the *P. vivax* PVP01 genome were mined using OrthoMCL (Release-6.8)(Chen et al., 2006). Within the differentially expressed genes in *P. vivax*, transcripts encoding for cell fating RBPs were filtered for further assessment.

GO enrichment analyses

GO enrichment analysis was performed on the PlasmoDB (v55) website with the following input parameters selected: P value cutoff = 0.05; Limit to GO Slim terms = No; Evidence: Computed, curated. Enrichment from GO cellular compartment (CC), biological processes (BP), and molecular function (MF) were all assessed.

Assessment of host hepatocyte transcriptomes

For the analyses of the host, we removed all transcripts derived from *P. vivax*, leaving only the transcripts derived from hepatocytes. We retained transcripts detected in at least 30 cells. Next, cells with less than 1,500 UMIs were removed from further analyses. Post-cell and gene filtering, the data from each replicate were normalized using Seurat's 'LogNormalize' function with the default parameters.

Data integration and clustering

Filtered, normalized data matrices were integrated in a manner described in in the Seurat (version 4) vignette, *Introduction to scRNA-seq integration*, available on the Sajita Lab's website (https://satijalab.org/seurat/articles/integration_introduction.html). Briefly, integration features were identified in each replicate using the 'SelectIntegrationFeatures' function. Next, integration anchors were identified using the 'FindIntegrationAnchors'. Last, using these anchors, the four datasets were integrated using the 'IntegrateData' function. Clustering was performed using Seurat's SNN-Leiden algorithm (Traag et al., 2019).

Gene Ontology and Reactome enrichment analyses

Enriched biological processes and pathways were determined using gProfiler (Raudvere et al., 2019) with the following options selected: ordered query, statistical domain scope—only annotated genes, significance threshold—g:SCS threshold, user threshold—0.05, Gene Ontology—GO biological processes, No electronic GO annotations, biological pathways—Reactome. Transcripts that displayed significant changes in expression in infected hepatocytes versus non-infected hepatocytes were used as input. EnrichmentMap (v 1.1.0) (Merico et al., 2010) for CytoScape (v 3.8.2) (Shannon et al., 2003) was used to create the enrichment networks. Significantly enriched GO biological processes and Reactome

pathways (adjusted p value < 0.05) were used as input. Pathways were clustered and annotated using the AutoAnnotate function (Kucera et al., 2016). Node size corresponds to the number of transcripts in the corresponding set, and edge widths correspond to the number of transcripts shared between sets.

Transcription factor enrichment analysis

We downloaded curated sets of known transcription factors from the website (<http://amp.pharm.mssm.edu/Enrichr/>). Transcription factor—target gene sets were obtained by ChIP-X experiments from the ChEA (Lachmann et al., 2010) and ENCODE (Project Consortium, 2004) projects. TF-target relationships using Chip-seq data from ENCODE and CHEA were used to identify overrepresented pathways and TFs. Differentially expressed genes (adjusted p value < 0.05) in each condition versus non-infected cells were used as inputs. Gene sets with an adjusted p value < 0.05 were considered significantly enriched.

Carfilzomib dose-response assessment

A carfilzomib dose-response against *P. vivax* liver forms was obtained from three independent experiments, in which a different *P. vivax* clinical isolate was allowed to infect BGW human hepatocytes. The 12-day assay was used (Maher et al., 2021). All independent experiments resulted in sufficient hypnozoite formation within each well for dose-response calculations. Liver stage parasite growth metrics and compound dilutions were loaded into CDD Vault (Collaborative Drug Discovery) and growth data normalized such that zero (negative) inhibition was set as the average of DMSO wells and 100% (positive) inhibition was set to the effective doses of a nigericin control. Normalized values were then combined in Graphpad Prism to fit a dose-response curve and calculated IC₅₀ values from all replicates.

Quantification of AKR1B10 protein expression

To analyze AKR1B10 expression and localization, *P. vivax*-infected PHHs were fixed 12 days post-infection. Infected wells were then permeabilized for 20 minutes with 0.2% TritonX-100, washed thrice with 1X PBS, and blocked in 3% BSA for one hour. Primary antibodies rabbit anti-HsAKR1B10 (PA5-30773, Thermo Fisher) and mouse anti-PvUIS4 were sequentially added, diluted 1:100 and 1:500, respectively. Antibodies were incubated overnight at 4°C and washed 3 times with 1X PBS before addition of 1:400 Alexa-Fluor 568 donkey anti-rabbit for HsAKR1B10 or 1:400 AlexaFluor 488 goat anti-mouse for PvUIS4. Secondary antibodies were incubated overnight at 4°C. Cells were washed thrice with 1X PBS before adding 0.5 mg/mL DAPI for 10 minutes. Cells were washed 3 final times with 1X

PBS before image collection with an ImageXpress inverted confocal microscope (Molecular Devices).
Image analysis was completed using MetaXpress analysis software (Molecular Devices).

Contact for reagent and resource sharing

Further information and requests for resources and reagents should be directed to and will be fulfilled
by the Lead Contact, Dennis Kyle (email: dennis.kyle@uga.edu).

Data availability

Raw sequencing files (FASTQ), processed data, scripts, and all host- and parasite- differentially
expressed genes will be made publicly available upon publication of the manuscript.

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SUPPLEMENTARY MATERIAL

Supplementary Table S1. Comparison of metrics derived from scRNA-seq and high content imaging data analyses.

Figure S1. Assessment of *P. vivax* liver stages using high-content imaging and scRNA-seq. (A) Representative high content images of primary hepatocytes infected with *P. vivax* on day 5 (left) and day 9 (right) post-infection. Images were obtained from one well from a 384-well plate with a 4x objective. Inset: one field of view (orange box) from the same well captured with a 20x objective. Cells were stained with DAPI (blue) and PvUIS4 (green). White arrow: liver form assigned as a hypnozoite, yellow arrow: liver form assigned as a schizont. White bar represents 1mm, grey bar represents 200µm. Images are representative of replicate 2. (B) Number of *P. vivax* liver stage transcriptomes assessed post-cell and gene filtering. (C) Number of *P. vivax* liver stage parasites assigned to cluster 1 and cluster 2 (hypnozoites and schizonts, respectively).

Figure S2. *P. vivax* schizonts and hypnozoites have distinct transcriptomic signatures. (A) Scatterplot showing average log₂ fold change versus the difference in the proportion of cells the transcript is detected in clusters encoding for hypnozoites and schizonts. Negative values: greater detection in hypnozoites; positive values: greater detection in schizonts. (B) Violin plots displaying the expression of genes encoding for TCA- and glycolysis-related proteins. (C) Dot plots showing transcripts with decreased expression in hypnozoites relative to schizonts. The size of the dot corresponds to the percentage of cells expressing the gene, colored by average expression. Differentially expressed transcripts were identified using Seurat's FindMarkers function. Wilcoxon rank-sum test, adjusted p values < 0.01. Scale: normalized expression; pct. exp: percent of cells expressing the gene. ldh: L-lactate dehydrogenase; gapdh: glyceraldehyde 3-phosphate dehydrogenase; atp-synthC: ATP synthase subunit C; gdh1: NADP-specific glutamate dehydrogenase 1.

Figure S3. Carfilzomib inhibits *P. vivax* schizonts and hypnozoites. (A) Violin plots displaying the distribution of expression of select genes encoding for proteasome subunits in schizonts and hypnozoites. (B) Table of proteasome-associated transcripts differentially expressed between schizonts and hypnozoites. Positive average log₂FC: higher expression in schizonts. (C) Structure of carfilzomib. (D) Dose-response curve of carfilzomib-induced inhibition of *P. vivax* schizont growth area (yellow, IC₅₀ 288nM), hypnozoite quantity per well (blue, IC₅₀ 511nM), and hepatocyte nuclei quantity (orange, IC₅₀ 3.32 µM). Data shown are pooled from three independent experiments, each containing duplicate wells at each concentration. Bars represent ± SEM.

Figure S4. Assessment of hypnozoite transcriptomes. (A) UMAP of hypnozoites colored by cluster and faceted by day post-infection. (B) Violin plot showing the average log₂FC of differentially expressed genes in each cluster. Markers were identified using Seurat's AllFindMarkers function. Transcripts with an adjusted p value < 0.05 (Wilcoxon rank-sum) and average log₂FC > 0.5 were deemed markers. Red dashed line: average log₂FC = 0.5.

Figure S5. Analysis of host-pathogen transcriptional signatures in *P. vivax*-infected and non-infected hepatocytes. (A) Number of hepatocytes assessed. Colored by infection status and faceted by sample (left); proportion of infected, exposed, or naive hepatocytes assessed on day 5 or day 9 post-infection. (B) t-SNE plot of hepatocytes colored by day. (C) t-SNE plots of hepatocytes colored by percent expression of zonation markers in human hepatocytes. Markers obtained from Macparland et al. (2019). (D) t-SNE plots of hepatocytes colored by hypnozoite infection status. Grey, uninfected (naive and exposed) hepatocytes; red, hepatocytes infected with C1 hypnozoites; blue, hepatocytes infected with C2; teal, hepatocytes infected with C3 hypnozoites. (E) Unique and overlapping gene sets in hepatocytes infected with schizonts or hypnozoites. Positive enrichment derived from genes displaying greater expression versus non-infected hepatocytes; negative enrichment derived from transcripts displaying decreased expression versus noninfected hepatocytes.

Figure S6. Human AKR1B10 is upregulated in hepatocytes infected with *P. vivax*. (A) Representative confocal images of *P. vivax* parasites on day 12 post-infection of hepatocytes. Cells were stained with DAPI (blue), PvUIS4 (green), and HsAKR1B10 (red). White bar represents 100µm. (B) Violin plots displaying the distribution of *AKR1B10* transcript levels in infected (schizonts and hypnozoites) and non-infected (exposed and naive) hepatocytes. Black dot: mean expression (log₂). (C) Violin plot displaying the distribution of AKR1B10 signal in hepatocyte cultures infected with *P. vivax*. (D) Associated with panel C, violins plots displaying the distribution of AKR1B10 signal across biological replicates. IHC: immunohistochemistry; SZ: hepatocyte containing schizont; HZ: hepatocyte containing hypnozoite; EX: hepatocyte not infected but exposed to *P. vivax*. *****: p ≤ 0.0001; *: p ≤ 0.05; n.s: not significant, p > 0.05.

Data Set S1. Data associated with schizonts versus hypnozoites analyses. (A) Differentially expressed genes defining schizonts and hypnozoites. Positive Average log₂ FC: Upregulated in hypnozoites relative to schizonts. (B) Differentially expressed genes identified as encoding for cellular fating RNA-binding proteins. (C) GO Term enrichment of Hypnozoite or Schizont transcripts identified as cellular fating RBPs. Rows highlighted in light green are terms with Bonferroni adjusted p value < 0.05. Input parameters: P value cutoff = 0.05; Limit to GO Slim terms = No; Evidence: Computed, curated; PlasmoDB v55.

1019 **Data Set S2.** Data associated with analyses of hypnozoite-to-hypnozoite heterogeneity. (A) Markers
1020 defining each hypnozoite cluster. Marker defined as: Average log₂ FC > 0.5, Adjusted p value < 0.05.
1021 Rows colored green are transcripts that meet marker criteria. (B) Differentially expressed genes
1022 identified as encoding for cellular fating RNA-binding proteins in hypnozoite clusters. (C) GO Term
1023 enrichment of hypnozoite transcripts identified as cellular fating RBPs. Rows highlighted in light green
1024 are terms with Bonferroni adjusted p value < 0.05. P value cutoff = 0.05; Limit to GO Slim terms = No;
1025 Evidence: Computed, curated; PlasmoDB v55.

1026 **Data Set S3.** Data associated with host-specific transcriptional signatures in response to *P. vivax*
1027 infection. (A) Differentially expressed genes in hepatocytes containing schizonts versus non-infected
1028 cells. Positive average log₂FC: greater expression in infected hepatocytes. (B) Differentially expressed
1029 genes in hepatocytes containing hypnozoites versus non-infected cells. Positive average log₂FC: greater
1030 expression in infected hepatocytes. (C) Enrichment of GO biological processes and Reactome metabolic
1031 pathways. (D) Unique and overlapping enriched gene sets in hepatocytes infected with schizonts or
1032 hypnozoites. (E) Transcription factor enrichment analysis. Rows highlighted in green are sets that are
1033 significantly enriched, adjusted p value < 0.05.

Figure 1

A

i

High content imaging

ii

scRNA-seq

B

Day 5 post-infection

Day 9 post-infection

C

Replicate 1

Replicate 2

Figure 1. Design and validation of detection of *P. vivax* liver stages using high-content imaging and scRNA-seq. (A) Schematic illustrating the sample preparation and data generation pipeline used to assess *P. vivax* liver stage infection rates and gene expression. (B) Representative high content images of primary hepatocytes infected with *P. vivax* on day 5 (left) and day 9 (right) post-infection (p.i). Images were captured from one well from a 384-well plate with a 4x objective. Inset: one field of view (white dashed box) from the same well captured with a 20x objective. Cells were stained with DAPI (blue) and PvUIS4 (green). White bar represents 1mm, inset bars represent 10µm. Images are representative of replicate 1. (C) Histogram displaying the distribution of parasite growth areas fixed at day 5 or day 9 post-infection (left) and net quantity of parasites and hepatocyte nuclei (right). Net infection rate is indicated for each endpoint. Growth and infection metrics are shown for both replicates.

Figure 2

Figure 2. Low dimensional representation of *P. vivax* schizont and hypnozoite transcriptomes (A) t-SNE plots of integrated *P. vivax* liver stage scRNA-seq data faceted by parasites isolated on day 5 (left) and day 9 (right) post-infection. (B) t-SNE plot of *P. vivax* liver stage data colored by parasites encoding for putative hypnozoites (C1) and schizonts (C2). (C) t-SNE plots of *P. vivax* liver stage scRNA-seq data colored by number of transcripts detected (left), UMI counts (middle), and the expression of the schizont marker, LISP2 (PVP01_0304700) (right). Scales: absolute count (left, middle), normalized expression (right).

Figure 3

Figure 4

Figure 4. Transcriptomic signatures of reactivating and persister hypnozoites. (A) Number of hypnozoites assessed (left) and UMAP of cells identified as hypnozoites colored by day of collection. (B) Proportion of cells in clusters on days 5 and 9 post-infection (left) and UMAP of hypnozoites colored by cluster. (C) Violin plot showing the expression of LISP2 in each cluster. (D) Dot plot showing markers that distinguish hypnozoites in each cluster. The size of the dot corresponds to the percentage of cells expressing the gene of interest, colored by scaled average expression of the transcript in each cluster. Marker transcripts were identified using Seurat's AllFindMarkers function. Wilcoxon rank-sum test, average $\log_2FC > 1$, adjusted p value < 0.01 . (E) Number transcripts in each cluster encoding for cellular fating RBPs sorted by their average $\log_2FC$ relative to the other clusters (top) and GO Term enrichment of transcripts in each cluster (bottom). Adj P: Bonferroni adjusted p value.

Figure 5

Figure 5. Analysis of host-pathogen transcriptional signatures in *P. vivax*-infected and non-infected hepatocytes. t-SNE plots of hepatocytes faceted by infection status. Grey, noninfected (naive and exposed) hepatocytes; light blue, hepatocytes infected with schizonts; teal, hepatocytes infected with hypnozoites.

Figure 6

Figure 6. Analysis of host transcriptional signatures in *P. vivax* infected hepatocytes. (A) Volcano plots showing changes in gene expression in infected hepatocytes versus non infected (Wilcoxon rank sum, adjusted p value < 0.05). Positive fold change (FC) values represent genes with greater expression, and negative FC values represent genes with decreased expression. Dashed horizontal red lines: adjusted p value = 0.05; dashed vertical red lines: log₂FC = 0.5. (B) Summary of differential gene expression data shown in the volcano plots and of the enrichment analyses. Hep: hepatocyte. (C) Enrichment map of cellular processes and pathways associated with hepatocyte infection with *P. vivax* schizonts. (D) Enrichment map of cellular processes and pathways associated with hepatocyte infection with *P. vivax* hypnozoites. For the maps depicted panels C and D, node size is proportional to the number of genes identified in each gene set (minimum 3 genes/gene set); each node represents a distinct biological process or pathway derived from gene with decreased expression (red) or increased expression (blue) versus non-infected cells; and edges (blue lines) represent the number of genes overlapping between two processes or pathways.

Figure 7

Figure 7. Transcription factor enrichment in hepatocytes infected with schizonts or hypnozoites. Dot plot of transcription factors enriched in Hepatocyte^{+hypnozoite} (left) or Hepatocyte^{+schizont} sets (right). Positive (+) enrichment derived from genes displaying greater expression versus non-infected hepatocytes; negative (-) enrichment derived from genes displaying decreased expression versus noninfected hepatocytes. ENCODE (Feingold et al., 2004) and ChEA (Lachmann et al., 2010) consensus transcription factors from ChIP-X (downloaded from Enrichr (Kuleshov et al., 2016)).