

1    TITLE

2    Gene signatures and host-parasite interactions revealed by dual single-cell profiling of *Plasmodium vivax*  
3    liver infection

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30    SUMMARY

Malaria-causing *P. vivax* parasites can linger in the human liver for weeks to years, and then reactivate to cause recurrent blood-stage infection. While an important target for malaria eradication, little is known about the molecular features of the replicative and non-replicative states of intracellular *P. vivax* parasites, or the human host-cell responses to them. Here, we leverage a bioengineered human microliver platform to culture Thai clinical isolates of *P. vivax* in primary human hepatocytes and conduct transcriptional profiling of infected cultures. By coupling enrichment strategies with bulk and single-cell analyses, we captured both parasite and host transcripts in individual hepatocytes throughout the infection course. We defined host- and state-dependent transcriptional signatures and identified previously unappreciated populations of replicative and non-replicative parasites, sharing features with sexual transmissive forms. We found that infection suppresses transcription of key hepatocyte function genes, and that *P. vivax* elicits an innate immune response that can be manipulated to control infection. Our work provides an extendible framework and resource for understanding host-parasite interactions and reveals new insights into the biology of malaria dormancy and transmission.

44    KEYWORDS

*Plasmodium vivax*, hypnozoites, dormancy, hepatocytes, single-cell, transcriptomics, host-parasite  
45    interactions

46

50 INTRODUCTION  
51

52 *Plasmodium* parasite, the causative pathogen of malaria, has a complex life cycle that spans multiple hosts.  
53 Disease transmission is initiated upon bite of an infected *Anopheles* mosquito, which deposits infective  
54 parasites, called sporozoites, into the human host. Sporozoites travel to the liver, invade hepatocytes and  
55 replicate, forming thousands of new parasites called merozoites, which eventually break out into the blood  
56 stream, cyclically invading erythrocytes and initiating clinical symptoms. Parasite transmission back to the  
57 mosquito is ensured by the development of sexual gametocyte forms during the asexual erythrocytic cycle  
58 that are taken up upon bite to restart the life cycle in the insect host. Uniquely, in the case of *P. vivax*, a  
59 subset of liver-stage parasites develops into dormant forms called hypnozoites, which can re-activate weeks  
60 to years after initial infection to cause relapsing disease. Thus, the liver-stage, which is obligate yet  
61 clinically silent and includes relapse-causing hypnozoites, presents a unique opportunity for malaria  
62 intervention before onset of symptoms. However, our knowledge of liver-stage malaria, and the response of  
63 its hepatocyte host is sparse due to difficult access to the parasite and lack of suitable human liver models.  
64 To date, much of our historical knowledge has been based on liver biopsies of infected patients, making it  
65 challenging to perform mechanistic studies on liver-stage forms, especially the quiescent hypnozoites.  
66 Transcriptomic studies hold promise for unveiling mechanistic insight into liver-stage *P. vivax* relapsing  
67 biology, but the low infection rate and the reduced quantity of parasite transcripts in a transcriptionally  
68 active host cell environment has made it difficult to perform these studies.

69 We recently provided some of the first insights into the transcriptional features of *P. vivax* liver-  
70 stages by leveraging an *in vitro* primary human liver platform (MPCC, micropatterned co-cultures) that  
71 recapitulates key aspects of *P. vivax* liver-stage biology, including establishment of persistent dormant  
72 forms, growing schizonts, merozoite release, and subsequent infection of overlaid erythrocytes. Our work  
73 revealed reduced transcriptional activity in hypnozoite-enriched samples, specifically, suppressed  
74 transcripts for functions related to cell division and invasion machinery, consistent with a quiescent state  
75 (Gural et al., 2018). However, the single time-point bulk sequencing used in this study prevented us to  
76 capture the inherent heterogeneity of the distinct liver-stage parasite forms. Achieving a deeper  
77 understanding of pathogen-host interactions has the potential to provide insight into mechanisms that could  
78 be leveraged to treat or prevent infection, as suggested by innate interferon responses to infection by rodent  
79 malaria parasites (Liehl et al., 2013) and a number of viruses (Schneider et al., 2014). However, a closer  
80 look into infection-specific host responses and potential protective responses in uninfected neighbors  
81 requires single-cell resolution. Recently, diverse single-cell technologies have revealed stage-specific  
82 transcriptional signatures in mosquito (Real et al., 2021), blood and gametocyte (Poran et al., 2017; Walzer  
83 et al., 2018) stages from non-relapsing human parasites, and the entire life cycle of rodent parasites  
84 (Howick et al., 2019), all of which can be easily cultured and propagated in laboratories. For *P. vivax*,  
85 single-cell transcriptomic studies have been conducted in blood-stages collected from infected monkeys (Sà  
86 et al., 2020), but analysis of liver infection has not been performed to date.

87 Here, we present the first comprehensive view of the liver-stage transcriptomes of a human-  
88 infecting malaria parasite and its host cells at single-cell resolution. To achieve this goal, we coupled  
89 MPCC with Seq-Well, a recently developed low-cost and portable single-cell platform that does not require  
90 fluorescent labeling and is compatible with use of samples collected in endemic settings (Gierahn et al.,  
91 2017; Hughes et al., 2020). With this combined platform, we define distinct signatures between early,  
92 dormant, mid, and late-stage parasites, and identify a sub-population of sexually committed forms in the  
93 liver, previously thought to appear only during erythrocytic infection. We interrogate pathogen-host  
94 interactions and describe innate immune responses by uninfected bystander cells, representing a likely  
95 mechanism for endogenous protection from secondary infections. We validate expression of host  
96 candidates and report cytokine- and stage-dependent anti-parasite activity by induction of interferon  
97 signaling. Together, the data presented here provide a closer look at transcriptional signatures in *P. vivax*  
98 liver-stages, including host responses, and offer novel insights into their unique biology.

100 RESULTS

101

102 **Profiling liver-stage *P. vivax* infection by pairing targeted sequencing with single-cell analysis**

103 To comprehensively profile *P. vivax* liver-stage infection, we collected cells from MPCC cultures of  
104 primary human hepatocytes at multiple time points following infection (**Figures 1A-B, S1A**). Sampling  
105 spanned the full liver-stage developmental period (days 1 to 11), comprising a mix of both replicating  
106 schizonts and non-replicative hypnozoites. To obtain hypnozoite-enriched samples, MPCCs were treated  
107 from day 5 to 8 with a phosphatidylinositol 4-kinase (Pi4K) inhibitor compound, a dosing regimen that  
108 eliminates the replicative parasites while preserving the dormant parasites (Gural et al., 2018). For  
109 hypnozoite-enrichment without drug treatment, we collected cultures at day 14. To obtain a baseline  
110 reading of the host response, naïve and mock-exposed MPCCs were prepared and collected in parallel. A  
111 total of 56 samples from two independent infections performed with clinical Thai *P. vivax* isolates were  
112 processed for high throughput single-cell RNA sequencing (scRNA-seq) using Seq-Well S<sup>3</sup> (Hughes et al.,  
113 2020). Samples were also collected in bulk for RNA sequencing (RNA-seq) analysis (**Figure 1A**).

114 Because whole transcriptome scRNA-seq resulted in poor representation of *P. vivax* genes and  
115 transcripts, we incorporated an additional step whereby barcoded parasite transcripts were enriched using  
116 previously validated nucleic acid baits targeting the entire *P. vivax* genome (Gural et al., 2018). Capture  
117 and re-sequencing of samples increased the efficiency of *P. vivax* gene and transcript detection by 5.6-fold  
118 per single parasite (**Figures 1C, S1B**). Combining single-cell transcriptomes from pre- and post-capture,  
119 we recovered 1,991 individual parasites with greater than 10 genes or 100 *P. vivax*-mapped transcripts. This  
120 tally fairly reflects the number of hepatic infections at the different timepoints, except for day 11 samples  
121 from which a higher number of parasites was recovered (likely representing free merozoites released from  
122 mature schizonts during sample processing; **Figure S1A; Table S1**).

123

124 **Single-cell profiling *P. vivax* liver infection defines stage-specific gene signatures**

125 Integration of parasite transcriptomes with Scanorama yielded 8 *P. vivax* clusters, corresponding to distinct  
126 developmental liver-stages that were visualized by uniform manifold approximation and projection  
127 (UMAP; **Figures 1D-E**). Cluster Pv\_C1 contains early-stage individual parasites (day 1), while clusters  
128 Pv\_C2-C4 comprise *P. vivax* parasites present in the mixed samples collected on days 4 to 8, as well the  
129 hypnozoites from Pi4K-enriched cultures and the day 14 samples. Late-stage schizont parasites (day 11) are  
130 scattered across clusters Pv\_C5-C8.

131 Based on the gene patterns that define each cluster (**Figure S2A; Table S1**), early liver-stage  
132 parasites (Pv\_C1) are characterized by residual expression of sporozoite-specific genes and expression of  
133 genes involved in cytoskeleton organization process, likely necessary to establish intracellular infection.  
134 Pv\_C2-C4 clusters appear to represent a core mid-stage liver program, comprising genes implicated in  
135 housekeeping functions important for parasite growth such as translation (*EIF1D,G; EIF2A,B; EIF3A-E*)  
136 and metabolism (*ENO, ACC, HCS1*). Well-characterized genes such as those encoding liver-specific  
137 protein 1 (*LISP1*) and 2 (*LISP2*) appear as top marker genes for these clusters. Progressing along the liver-  
138 stage development, GO terms for cell cycle, mitotic division, and adhesion of symbiont to host become  
139 significantly represented in Pv\_C5-C6. Gene cluster identifiers include several members of merozoite  
140 surface proteins (*MSP*), serine repeat antigen (*SERA*), rhoptry associated (*RAP, RAMA*) and rhoptry neck  
141 (*RON*) multigene families that are necessary for red blood cell invasion, and also the merozoite egress  
142 subtilisin-like protease 1 (*SUB1*). Finally, among the late-stage parasites Pv\_C7-C8 represent a previously  
143 unidentified population of hepatic parasites that co-express merozoite- and gametocyte-specific genes.  
144 Marker genes for these clusters include multiple copies of the tryptophan-rich protein family, which are  
145 expressed by merozoites of multiple *Plasmodium* species and have been implicated in erythrocyte invasion,  
146 the gamete antigen *G27/25*, the gamete release protein (*GAMER*) and the homolog of gametocyte  
147 exported protein 5 (*GEXP5*, aka *PHISTc*).

148

149 **Transcriptional profiling of *P. vivax* liver-stages reveals early sexual commitment**

150 To gain further insight into the subpopulation of parasites defined by clusters Pv\_C7 and Pv\_C8, we  
151 searched the dataset for additional gametocyte-specific genes. Expression of canonical sexual markers  
152 (*PVS16*, *P28*, *P230*), including male (*MGET* and *MDG1*) and female (*RUBVI* and *G377*) specific genes  
153 recently assigned to *P. vivax* (Sà et al., 2020), was detected throughout the developmental liver-stages in  
154 multiple clusters (**Figure 2A**). Their expression was also confirmed via targeted bulk RNA-seq (**Figure**  
155 **2B; Table S2**) and by RT-qPCR analysis of a small subset of genes (**Figure 2C**). For further validation, we  
156 performed *in situ* hybridization for *GEXP5*, which appears highly expressed in day 11 samples (**Figures**  
157 **2B, 2C**) and is known as an early sexual stage marker in blood-stages of the human malaria parasite *P.*  
158 *falciparum* (Tibúrcio et al., 2015). We found *GEXP5* transcripts in 5-15% of schizonts, with positive signal  
159 in all merozoites, while the remaining schizonts did not contain this transcript (**Figure 2D**). Interestingly,  
160 we found that 16-25% of parasites in clusters Pv\_C1 and Pv\_C6 (**Figure 2A; Table S1**) express the gene  
161 encoding AP2-G, the transcriptional master regulator of sexual development in blood stages (Kafsack et al.,  
162 2014; Sinha et al., 2014). Exploring the dynamics of this transcription factor, we found induced expression  
163 of *AP2-G* and its upstream activator, gametocyte development 1 (*GDVI*) (Filarsky et al., 2018), as early as  
164 day 1 after hepatocyte invasion. Expression of *PVS16*, a downstream target of AP2-G increased from day 2,  
165 and onwards (**Figure 2E**). *PVS16* protein has been detected in a fraction of *P. vivax* hepatic schizonts at  
166 day 8 (Roth et al., 2018; Schafer et al., 2020). Altogether, the data indicates that commitment to  
167 gametocytogenesis might occur early during liver-stage development in a subset of parasites, likely leading  
168 to formation of sexually committed merozoites.

169

170 ***P. vivax* non-replicative liver-stages depend on proteolytic activity and are sexually committed**

171 To inform the identification of hypnozoite-specific gene signature(s), we re-clustered the mid-stage parasite  
172 clusters Pv\_C2-C4 (**Figures 3A-B; Table S1**). This enabled us to explore transcriptional differences among  
173 the mixed and hypnozoite-containing samples and revealed a sub-cluster (Pv\_SC3) significantly enriched in  
174 genes encoding proteins with peptidase activity (10 genes) and nucleic acid binding proprieties (18 genes)  
175 in the non-replicative parasite population. These include several members of protease families (vivapains  
176 and plasmepsins), members of ApiAP2 family of transcription factors (*AP2-Tel* and *AP2-FG*) and *PUF1*, a  
177 Pumilio RNA binding protein known to be involved in translational repression (Bennink and Pradel, 2019).  
178 Consistent with a quiescent state, parasites in Pv\_SC3 show low levels of the replicative marker *LISP2*  
179 (**Figure 3C**). The expression of transcriptional regulators of gametocytogenesis *AP2-G* and *AP2-FG*  
180 prompted us to look for additional gametocyte-related genes (as in **Figure 2A**). Remarkably, we found  
181 expression of canonical sexual markers (*P28*, *P25*, *P230*) in Pv\_SC3 subcluster, suggesting that a  
182 subpopulation of *P. vivax* quiescent hypnozoites could be pre-committed to become sexually transmissive  
183 forms (**Figures 3C-D**). A larger number of gametocyte-specific genes however was found in the Pv\_SC5  
184 subcluster, which is largely *LISP2* positive. Thus, Pi4K-treated and day 14 parasites in Pv\_SC5 subcluster  
185 likely represent a subpopulation of sexually committed reactivating hypnozoites. Presence of gametocyte-  
186 specific transcripts in Pi4K-treated samples was confirmed by RT-qPCR analysis, further supporting the  
187 concept that non-replicative parasites could be sexually committed (**Figure S3A**).

188 Protein degradation is important for maintenance of a hibernatory state induced by nutrient  
189 limitation during the *P. falciparum* erythrocytic cycle (Babbitt et al., 2012). To determine whether  
190 proteolytic activity could be required to maintain viability of quiescent hypnozoites, we exposed *P. vivax*-  
191 infected MPCCs to E64, a membrane-permeable protease inhibitor. No effect was found on schizont forms,  
192 however, the number of *P. vivax* hypnozoites was significantly reduced in the E64-treated cultures (**Figures**  
193 **3E, S3B**). Altogether the results suggest that *P. vivax* hypnozoites in the liver could represent a reservoir of  
194 sexually committed parasites and dormancy might depend on transcription and translational repression  
195 mechanisms and proteolysis for long-lasting viability.

196 **Pre-erythrocytic sexual differentiation is associated to distinct host metabolic states**

197 To characterize host cell signatures linked to infection and host-dependent differences in parasite  
198 transcriptomes, we profiled samples of unexposed, mock- and *P. vivax*-exposed MPCCs across all  
199 timepoints. Integrating the data from 31,767 high-quality human transcriptomes with Seurat revealed 8  
200 human clusters, likely representing diverse hepatic cell states (**Figures 4A, S4A**). Automated annotation of  
201 the clusters using SingleR tool identified clusters H0 to H3 as hepatocytes, exhibiting canonical markers of  
202 hepato-specific functions (**Figure S4B**). Albeit in small numbers, the remaining clusters H4 to H7 appear to  
203 include other cell types besides hepatocytes, such as endothelial and immune cells, likely present in the  
204 primary human liver sample used to generate the MPCC. Notably, the cells in the outlying cluster H8  
205 express classical immune markers such as *CD45*, *CD4* and *CD32*.

206 *P. vivax*-associated hepatocytes were embedded throughout the cluster landscape, without a clear  
207 segregation between infected and non-infected cells (**Figure 4B**). Nevertheless, comparing *P. vivax*-  
208 positive and -negative cells in general demonstrated that infected cells are transcriptionally distinct  
209 (**Figures 4C, S4C; Table S3**). Gene set enrichment analysis revealed suppression of immune defense and  
210 liver-specific functions and increased expression of genes related to membrane organization and  
211 modulation by symbiont of host cellular process in *P. vivax*-infected cells (**Figure S4C**). Examples of  
212 differentially expressed genes include markers of inflammation and stress response, such as the family of  
213 acute-phase serum amyloid A proteins (*SAA4*) and the NFKB inhibitor alpha, caspases and apoptosis  
214 regulators (*BIRC3* and *IFI6*), and a glucose transporter (*SLC2A2*) with increased expression in the *P. vivax*-  
215 infected population. In contrast, interferon (IFN) regulatory factors (*IRF7*) and IFN induced proteins (*MX1*)  
216 are expressed at low levels in *P. vivax*-positive cells. Notably, *EPHA2* was found expressed in higher levels  
217 in 50% of the infected cells, consistent with a recent report identifying it as a host factor for human-  
218 infecting *P. falciparum* and rodent parasites (Kaushansky et al., 2015). Comparing infected hepatocytes  
219 that harbored mid- and late-stage parasites, we found IFN-related genes with significant increased  
220 expression in late infection, such as *ISG15*, *IFIT1* and *IFI6*, which might be involved in creating a  
221 permissive environment for parasite late-stage development and replication (**Figure 4D; Table S3**).

222 Given that sexual differentiation and gametocytogenesis in malaria parasites have been linked to  
223 host-derived physiological signals (Brancucci et al., 2017), we next compared the transcriptomes of  
224 hepatocytes hosting *P. vivax* parasites expressing the transcription factor *AP2-G* versus *AP2-G* negative.  
225 Interestingly, we found that hepatocytes bearing *AP2-G* positive parasites exhibit significant lower  
226 expression of genes encoding proteins implicated in intracellular iron storage (*FTL* and *FTH1*) and lipid  
227 metabolism (*APOA2*, *APOC1*, *APOC3*, *FABP1*, *CYP3A4*). Genes encoding albumin (*ALB* and *TTR*) and  
228 some mitochondrial proteins are also differentially expressed (**Figure 4E; Table S3**). These observations  
229 suggest that deficiency in specific intracellular metabolites (possibly iron or lipids) could serve as trigger  
230 for *AP2-G* induction and pre-erythrocytic sexual commitment.

231

232 **Interferon responses in uninfected bystander hepatocytes**

233 Taking advantage of our single-cell approach, we also investigated the impact of *P. vivax* infection in the  
234 neighbor hepatocytes. Comparing the transcriptomes of cells exposed to *P. vivax* to mock-exposed or naïve  
235 hepatocytes, we detected a widespread induction of the alpha/beta IFN response, mainly in the *P. vivax*-  
236 exposed uninfected cells (**Figures 4F-H**). Day 1 samples stand out given that the induction of multiple  
237 IFN-responsive genes and gene families, including *IRF7*, *IFI6*, *IFI27*, *IFIT1-5*, *IFI44* and *IFIH1* is  
238 observed in more than 40% of the hepatocytes (**Figure 4F**). Transcripts of effector IFN-induced  
239 transmembrane proteins *IFITM2* and *IFITM3* are increased throughout the infection time course (**Figure**  
240 **4F**). The observed temporal order and gene composition of the IFN response to *P. vivax* is remarkably  
241 similar to that of hepatitis C virus (HCV) infection (Sheahan et al., 2014), suggesting a common hepatocyte  
242 defense mechanism in response to hepatotropic pathogens.

243 To validate our findings with an independent approach, we performed immunofluorescence  
244 analysis of *IFITM3* in *P. vivax*-infected MPCCs at a late infection time-point. We found that *IFITM3*

245 protein localizes in the bile canaliculi and tight junctions and confirmed the upregulation of IFITM3 in the  
246 uninfected bystander cells, with higher expression detected in the vicinity of infected hepatocytes (**Figures**  
247 **5A-B**). IFITM3 has recently been shown to enhance the trafficking of virus particles to lysosomes (Spence  
248 et al., 2019), and thus could be involved in targeting parasites for degradation. Based on its localization,  
249 IFITM3 could also be involved in gap junction communication and propagation of host responses from  
250 infected cells to uninfected neighbors (Luther et al., 2020; Patel et al., 2009).

251

### 252 **Host interferon responses control *P. vivax* infection**

253 Given the induced innate immune responses observed during early *P. vivax* infection, we hypothesized that  
254 the activation of this pathway could be associated with the significant drop in parasite numbers observed  
255 from day 1 to 11 (**Figure S1A**). To assess the impact of activated IFN signaling on the progression of *P.*  
256 *vivax* infection in MPCCs, we treated infected cultures with IFN alpha (IFNa) and beta (IFNb) cytokines.  
257 As control, we used IFN gamma (IFNg), which is known to reduce *P. vivax* infection (Boonhok et al.,  
258 2016; Ferreira et al., 1986). While no effect was detected in terms of the kinetics or frequency of parasite  
259 development (**Figure S5**), the number of both *P. vivax* schizonts and hypnozoites were significantly  
260 reduced upon treatment with IFNa (**Figure 5C**). IFNb appeared to have a stronger effect on schizont stages,  
261 by contrast IFNg treatment revealed a potent anti-hypnozoite activity, which is shown here for the first  
262 time. Altogether, the results suggest that successful *P. vivax* infection likely involves robust mechanisms  
263 for subverting host IFNa/b responses, which might be long-lasting in the hepatocytes that harbor dormant  
264 hypnozoites.

265

266

## 267 DISCUSSION

268

269 Here we comprehensively surveyed the molecular composition of *P. vivax* parasites in distinct liver-stages,  
270 as well as their host or neighboring hepatocytes, throughout the course of infection, providing the first  
271 single-cell liver atlas of relapsing human malaria. Leveraging the portability of two established platforms  
272 (MPCC and Seq-Well) to culture and collect individual human hepatocytes infected in an endemic location,  
273 we performed dual single-cell sequencing analysis and further developed a method to selectively enhance  
274 capture of parasite transcripts. We demonstrated the robustness and utility of this approach by sequencing  
275 1,991 *P. vivax* parasites and 31,767 hepatocyte transcriptomes and provided validations and mechanistic  
276 insights for newly identified gene signatures and parasite-host interactions.

277

278 Dual transcriptional profiling of *P. vivax* infection revealed host- and stage-dependent gene  
279 expression patterns in both parasites and hepatocytes. Our data is consistent with the following model: upon  
280 invasion, a subset of *P. vivax* sporozoites in cells with reduced hepatic metabolism will activate *AP2-G*.  
281 Of those, a fraction will remain dormant, while the remaining portion will activate the schizogony program.  
282 Thus, at the point of egress, two populations of hepatic *P. vivax* merozoites will emerge from the liver: the  
283 asexual population developing into blood-stages, and the sexually committed population developing into  
284 gametocytes for mosquito transmission. This model, while distinct from the existing understanding of the  
285 *Plasmodium* life cycle, is in agreement with historical observations of rapid *P. vivax* transmission that  
286 occur before the onset of symptoms (Baker, 2010), and is consistent with the high abundance of gametocyte  
287 transcripts detected in blood collected from malaria patients (Adapa et al., 2019; Kim et al., 2019). In our  
288 model, parasites that do not activate schizogony, the hypnozoites, remain in a dormant state via  
289 transcriptional/translational repression mechanisms and rely on proteolytic activity to sustain viability.  
290 Bypassing the need for an asexual replication phase might represent an evolutionary imperative to preserve  
291 genome integrity, given the prolonged period between infection and transmission. From a clinical  
292 perspective, this model would advocate for the development of a novel “wake and kill” clinical approach;  
293 namely, to leverage drugs that enhance gametocyte commitment as a way to reduce the hypnozoite  
reservoir in the liver. Parasite-specific protease inhibitors could also be screened for anti-relapsing activity.

294                    Additionally, our work reveals dysregulation of IFN and inflammatory signaling pathways in *P.*  
295 *vivax*-infected hepatocytes. The reduced expression of IFN-responsive genes in infected cells suggests a  
296 protection mechanism for the parasite from host cell-mediated killing. In fact, induction treatments with  
297 IFN in our immune-cell free microliver system demonstrated anti-parasite activity against both schizonts  
298 and hypnozoites. Downregulation of IFN signaling may also contribute to under-activation of adaptative  
299 immune responses by antigen presenting cells, leading to parasite persistence in the organism. However,  
300 the mechanisms by which IFN suppression is achieved in infected cells or the impact of host genetics and  
301 host-dependent IFN responses, key determinants of clinical outcome in HCV infections (Sheahan et al.,  
302 2014), remain unknown. In contrast, we described upregulation of innate immune response pathways in  
303 uninfected bystander cells, similar to those observed in viral infections that help control spreading of the  
304 virus to neighboring cells (Kotliar et al., 2020; Sheahan et al., 2014). In the context of malaria, this  
305 phenomenon might prime or protect cells from a secondary infection, as suggested by the inhibition of  
306 malaria re-infection by IFN in a rodent model of *Plasmodium* (Liehl et al., 2013, 2015). Future studies  
307 employing spatial single-cell transcriptomics could reveal *P. vivax*-specific gene signatures and spatially  
308 heterogeneous responses in bystander cells.

309                    In summary, this study presents the first transcriptional description of individual *P. vivax* liver-  
310 stages, their host cells and uninfected bystander cells over the course of infection in human hepatocytes.  
311 Leveraging multiple single-cell technologies (sequencing, *in situ* hybridization, and immunofluorescence),  
312 we reveal earlier-than expected sexual commitment during *P. vivax* liver-stage development in both  
313 replicative and non-replicative parasites, and a dominant innate immune response that exhibits distinct  
314 signatures in infected and uninfected bystander hepatocytes. Taken together, these clinically relevant  
315 insights provide a framework for characterizing host-parasite interactions in *P. vivax* infections. We expect  
316 the methods described here to be applicable for profiling not only other *Plasmodium* parasites at various  
317 stages of the life cycle, but also other intracellular pathogens where low abundance of transcripts or host  
318 contamination make it difficult to perform single-cell studies.

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328 MATERIALS & METHODS

329

330 **Fibroblasts and primary cell cultures**

331 J2-3T3 male murine embryonic fibroblasts (gift of Howard Green, Harvard Medical School) were cultured  
332 at < 20 passages in medium comprising of Dulbecco's Modified Eagle Medium (DMEM, Corning), 10%  
333 (v/v) bovine serum (Thermo Fisher Scientific), and 100 mg/mL penicillin-streptomycin (Corning) and were  
334 kept at 37°C in a 5% CO<sub>2</sub> environment.

335 Cryopreserved primary human hepatocytes were purchased from BioIVT, a vendor permitted to sell  
336 products derived from human organs procured in the United States of America by federally designated  
337 Organ Procurement Organizations. Human hepatocytes (male donor, age 57) were maintained in DMEM  
338 with 10% fetal bovine serum (FBS, GIBCO), 1% ITS (insulin/transferrin/selenous acid and linoleic acid,  
339 BD Biosciences), 7 ng/mL glucagon (Sigma-Aldrich), 40 ng/mL dexamethasone (Sigma-Aldrich), 15 mM  
340 HEPES (GIBCO), and 100 mg/mL penicillin-streptomycin (Corning). Hepatocyte cultures were kept at  
341 37°C in a 5% CO<sub>2</sub> environment.

342

343 ***P. vivax* parasites**

344 *Anopheles dirus* mosquitoes were fed on blood collected from symptomatic patients attending malaria  
345 clinics in Tak, Songkla, and Ubon-Ratchathani Provinces in Thailand, confirmed positive for only *P. vivax*  
346 via microscopy and RT-PCR. Briefly, *P. vivax* infected blood was drawn into heparinized tubes and kept at  
347 37°C until processing. Infected blood was washed once with RPMI 1640 incomplete medium. Packed  
348 infected blood was resuspended in warm non-heat inactivated naive human AB serum for a final hematocrit  
349 of 50%. Resuspended blood was fed to laboratory reared female *Anopheles dirus* mosquitoes for 30  
350 minutes via an artificial membrane attached to a water-jacketed glass feeder kept at 37°C. Engorged  
351 mosquitoes were kept on 10% sugar at 26°C under 80% humidity at the designated insectary at the Mahidol  
352 Vivax Research Unit. Sporozoites were dissected from the salivary glands of infected mosquitoes 14-21  
353 days after blood feeding and pooled in DMEM supplemented with 200 mg/mL penicillin-streptomycin.

354

355 **MPCCs and *P. vivax* infection**

356 Primary human hepatocytes were seeded on collagen-micropatterned 96-well plates and surrounded with  
357 murine embryonic fibroblasts 3T3-J2s as detailed previously (March et al., 2015). For the scRNA-seq  
358 analysis, MPCCs were established using 3T3-J2s expressing an inducible apoptosis switch (Chen et al.,  
359 2020). MPCCs were infected with fresh sporozoites obtained through dissection of *P. vivax*-infected  
360 mosquitoes. For mock samples, MPCCs were exposed to material from non-infected mosquito salivary  
361 glands (matched number of dissected mosquitoes). Unexposed samples received the vehicle. For scRNA-  
362 seq, *P. vivax*-infected cultures were collected from duplicate wells, whereas mock and unexposed samples  
363 represent single wells for each time-point. For IFN treatments, MPCCs were infected in triplicate wells.

364

365 **Drug treatments**

366 To obtain hypnozoite-enriched samples, *P. vivax*-infected MPCCs were dosed with a schizont-specific drug  
367 Pi4K inhibitor (MMV390048, 1 μM) for 3 days starting on day 5 after infection. Similar dosing schedule  
368 was used for treatments with IFN alpha 2a (11100-1), beta (8499-IF-010) and gamma (285-IF-100) (all  
369 purchased from R&D Systems). Cytokine units tested are given in the figures. E64 treatment started at 4h  
370 post-infection after media washing the cultures.

371

372 **Seq-Well and hybrid capture**

373 For the scRNA-seq analysis, an updated Seq-Well protocol including a second-strand synthesis step was  
374 employed (Hughes et al., 2020). Briefly, MPCCs were first treated with AP20187 (0.5 μM) for 30 minutes  
375 at 37°C to partially deplete the fibroblast cells via apoptosis. After washing, the cultures were dissociated  
376 by Trypsin (0.25%) 5-minute treatment at 37°C. A suspension with 10-15,000 cells was then loaded onto a

377 functionalized-polydimethylsiloxane array preloaded with uniquely barcoded mRNA capture beads. After  
378 cells had settled into wells, the array was sealed with a hydroxylated polycarbonate membrane with a pore  
379 size of 10 nm, facilitating buffer exchange while permitting cell lysis, mRNA transcript hybridization to  
380 beads, and bead removal before proceeding with reverse transcription. The obtained bead-bound cDNA  
381 product then underwent Exonuclease I treatment to remove excess primer before proceeding with second-  
382 strand synthesis and PCR amplification.

383 To capture parasite reads from Seq-Well, full length cDNAs were amplified an additional 5 cycles using  
384 Kapa HiFi polymerase including a 3-minute extension time to increase concentration for capture. 200-300  
385 ng of cDNA was concentrated using a speedvac, reconstituted in 3.4  $\mu$ L water and hybridized for 32 hours  
386 as previously described (Gural et al., 2018). 10  $\mu$ L of capture material was amplified for 15 cycles  
387 following the standard protocol. Captured cDNA was then prepared into Illumina libraries using  
388 NexteraXT (Illumina). Final libraries were quality controlled using Fragment Analyzer (Agilent) and qPCR  
389 prior to Illumina sequencing (NextSeq500).

390

### 391 **Quantitative RT-PCR**

392 Total RNA from pooled triplicate wells of *P. vivax*-infected MPCCs was extracted with TRIzol (Thermo  
393 Fisher), DNase treated and purified using the RNeasy MinElute Cleanup Kit (Qiagen). cDNA synthesis  
394 was performed using SuperScript II (Thermo Fisher) and RT-PCR was carried out using PowerUp SYBR  
395 Green Master Mix (Applied Biosystems) in a Roche Light Cycler 480 Real-Time PCR Detection System  
396 according to the manufacturer's instructions. The primers used are listed in **Table S4**. Relative gene  
397 expression was calculated with the delta-delta Ct method, using PVP01\_1213400 as housekeeping gene.  
398

399

### **Immunofluorescence analysis**

400 *P. vivax*-infected MPCCs were fixed in ice-cold methanol or 4% paraformaldehyde (PFA), washed  
401 in phosphate-buffered saline (PBS) and stored at 4°C. Parasites were detected using *P. vivax*-specific  
402 antibodies (PvUIS4, PvBip and PvCSP) on methanol fixed cells as described in (Gural et al., 2018). For  
403 IFITM3 staining, PFA-fixed cells were permeabilized with 0.2% TritonX100 for 10 minutes at room  
404 temperature, washed in PBS and blocked with 2% bovine serum albumin (BSA) in PBS for 30 minutes at  
405 room temperature. IFITM3 rabbit monoclonal antibody (59212, Cell Signaling) was incubated overnight at  
406 4°C (1:100). Alexa-conjugated 488 secondary anti-rabbit antibody (1:1000) was incubated for 1 hour at  
407 room temperature, followed by nuclear staining with Hoechst. Images were captured on a Nikon Eclipse Ti  
408 or Zen-ApoTome inverted wide-field microscopes using 20x objectives. To quantify parasite size, the area  
409 of parasite defined by the PvUIS4 staining was measured using NIS-Elements Microscope Imaging  
410 Software and automatically converted to equivalent diameter.

411

### 412 **Fluorescence in situ hybridization**

413 *P. vivax*-infected MPCCs were fixed in 3.7% PFA for 10 minutes at room temperature, washed in PBS,  
414 immersed in 70% ethanol and stored at 4°C. Custom labelled probes set specific to *Pv18S* rRNA (FAM  
415 dye) and *PvGEXP5* (Quasar 670 dye) purchased from Stellaris were hybridized overnight in the dark at  
416 37°C following the manufacturer's instructions. After nuclear staining and washing, cells were imaged in a  
417 Nikon Eclipse Ti fluorescence microscope as described above.

418

### 419 **Sample Sizes and Statistical Analysis**

420 *n* represents the number wells from each plate as described in the figure legends. Exception for Figures 2C  
421 and 2E, where *n* represents 2 independent infections. Methods used for computing statistical significance  
422 are indicated in figure legends. Statistical significance was considered for p values below 0.05. Data was  
423 analyzed using GraphPad Prism Software.

424

425 **Bulk RNA-seq analysis**

426 fastq files were mapped using STAR v. 2.5.3a (Dobin et al., 2013) against PVP01 *P. vivax* v1 genome  
427 assembly and annotation, and quantitated by RSEM v. 1.3.0 (Li and Dewey, 2011). Differential expression  
428 analysis was performed in DESeq2 on protein-coding genes (Love et al., 2014).

429

430 **Tri-genome mapping target generation**

431 Chromosomes and contigs from human (hg19), murine (mm10) [same ENSEMBL releases as in (Macosko  
432 et al., 2015)] and *P. vivax* genome (PvP01 v1 release) were renamed with a species-specific prefix and  
433 concatenated into a fasta file. Corresponding gtf files were adapted to match prefixed chromosome names,  
434 and genes names were adjusted as an ENSEMBLID\_SPECIES\_GeneSymbol concatenated string.

435

436 **scRNA-seq processing**

437 For each sample, fastq files originating from multiple sequencing runs were concatenated and processed  
438 using an analytical pipeline derived from the DropSeq pipeline v. 1.12, as described in (Gierahn et al.,  
439 2017) (<https://github.com/broadinstitute/Drop-seq>). Briefly, reads were converted to a bam file using picard  
440 v. 2.9.0-1-gf5b9f50-SNAPSHOT, tagged with cell and transcript barcodes, and subsequently sequencing  
441 adapters and polyadenosine tracts were trimmed. Upon regenerating fastq files, reads were aligned with  
442 STAR v. 2.5.3a (Dobin et al., 2013) against the aforementioned tri-genome reference. Genomic features of  
443 the aligned reads were annotated using the combined species-gene symbol nomenclature including gene  
444 and exon of origin when relevant. Bead synthesis errors were assessed and when possible, altered unique  
445 molecular identifiers (UMIs) were repaired. Cell barcode abundance was tallied, and gene expression was  
446 called for the top 10,000 cell barcodes. Count matrices of genes x cells were imported in the R v. 3.6  
447 statistical environment and Seurat v.3 was used as the primary analytical package (Satija et al., 2015).  
448 Count matrices were merged into a single Seurat object, which was split by the sample of origin. Human,  
449 murine and *P. vivax* genes and transcripts counts were tallied for each cell barcode.

450

451 **scRNA-seq analysis of human transcriptomes**

452 Log10 ratios of human and murine-mapped transcripts per cell barcodes were calculated, and only cell  
453 barcodes with a ratio greater than 0 were retained. Dataset integration features were selected using Seurat's  
454 SelectIntegrationFeatures function, picking 3,000 genes and subsequently used to prepare the dataset for  
455 SCT-based integration PrepSCTIntegration). Integration anchors were identified using the  
456 FindIntegrationAnchor function, with parameters "SCT" as a normalization method, the aforementioned  
457 list of 3,000 genes as anchor features, 30 PCA dimensions, k.anchor=10 and using the first sample as  
458 reference (Butler et al., 2018). This integrated Seurat object was further filtered and cell barcodes  
459 displaying a human/murine coverage greater than 10-fold were retained for downstream analyses, resulting  
460 in 31,767 cells remaining. The integrated object was scaled and centered using a linear model, with  
461 scale.max=10, block.size =1,000 and min.cells.to.block=3,000. Principal component analysis was  
462 performed using RunPCA, retaining the top 30 components. The number of principal components retained  
463 was picked based on the inspection of the "elbow plot" and JackStraw procedure (n=100 replicates, 50  
464 PCs) in Seurat. A UMAP embedding was calculated on the top 30 principal components using the  
465 RunUMAP function with flags n.neighbors=30, metric=cosine, learning.rate=1, min.dist=0.3, spread=1,  
466 set.op.mix.ratio=1, local.connectivity =1, repulsion.strength =1, negative.sample.rate=5, uwot.sgd=  
467 FALSE, seed.use : 42, angular.rp.forest : FALSE on the integrated object. Cluster identification was based  
468 on the Louvain algorithm for nearest-neighbor identification: the top 30 principal components were used to  
469 build the kNN graph, considering 20 nearest neighbors, with prune.snn=0.067, nn.method was an exact  
470 RANN (nn.eps=0) and Euclidean distance as the annoy. The resulting graph was partitioned using a shared  
471 nearest neighbor (SNN) modularity optimization-based clustering algorithm at resolution 0.8 to identify  
472 clusters (with 10 starts and 10 iterations, and standard modularity function). Gene and transcript coverage  
473 plots per cell barcodes were generated on the integrated dataset prior to the final filtering step (for barcodes

474 with a 10-fold enrichment for human transcript). Cluster markers were identified using the FindAllMarkers  
475 function with logfc.threshold set to -100 and at least 10% of cells expressing the marker. Differential gene  
476 expression signature was queried between *P. vivax*-positive and -negative cells using a Wilcoxon test  
477 (implemented in the FindMarker function). Cell barcodes positive for eight or more parasite genes were  
478 deemed to be *P. vivax*-positive.

479

480 **scRNA-seq analysis of *P. vivax* transcriptomes**

481 The Seq-Well analytical pipeline was run on the samples as described above. For samples for which pre-  
482 and post-capture data were available, matching cell barcodes were identified in the corresponding genes x  
483 cell matrices and UMI counts were summed over pre- and post-capture libraries. The resulting matrices  
484 were merged into a Seurat object (2,171 cells, 85,276 genes). Only *P. vivax* genes were retained for  
485 subsequent analysis (n=6,478). Samples which had less than 20 cells were excluded from the analysis. The  
486 resulting object (n=1,991 cells) was split by dataset. Raw counts for each sample were exported into  
487 individual text files and processed with Scanorama (v. 1.5, under Python 3.6 environment) for  
488 normalization, batch correction and integration (Hie et al., 2019). The top 19 integration dimensions were  
489 imported back into Seurat and used for data embedding using UMAP, as well as cluster elicitation via the  
490 Louvain algorithm (similar to the procedure ran for the human compartment described above). Cluster-  
491 specific gene markers were identified using a Wilcoxon test. Subclustering was performed by  
492 extracting Scanorama dimensions for the cells of interest, followed by UMAP and Louvain-based cluster  
493 identification. Heatmaps were generated in Seurat using hclust-based hierarchical clustering of the scaled  
494 data with a centroid distance metric (UPMGC-equivalent), followed by manual editing.

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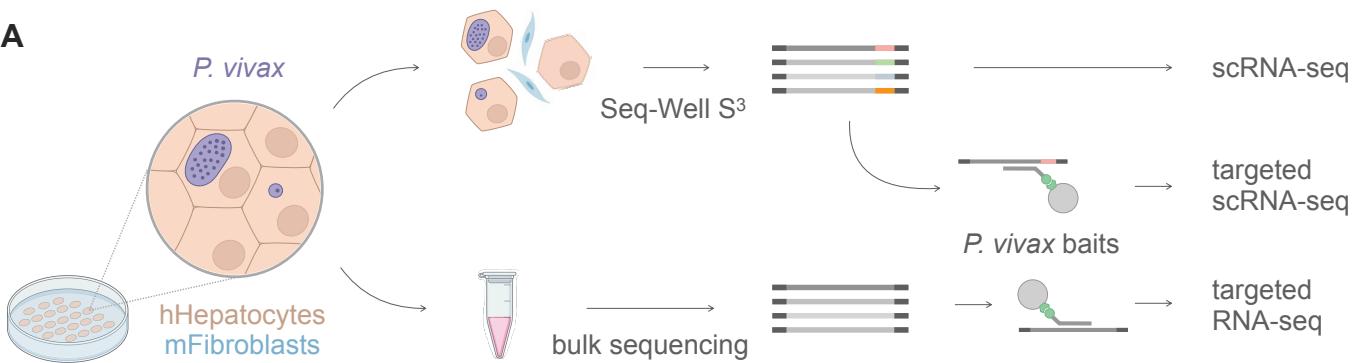
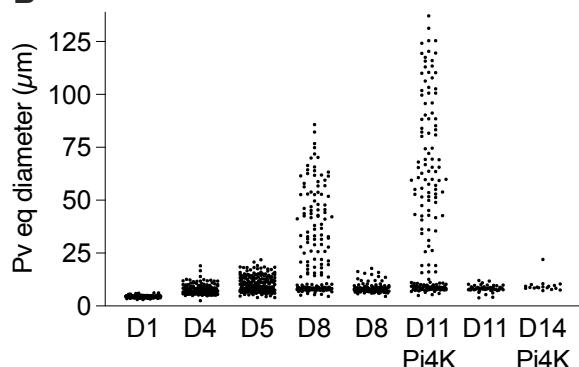
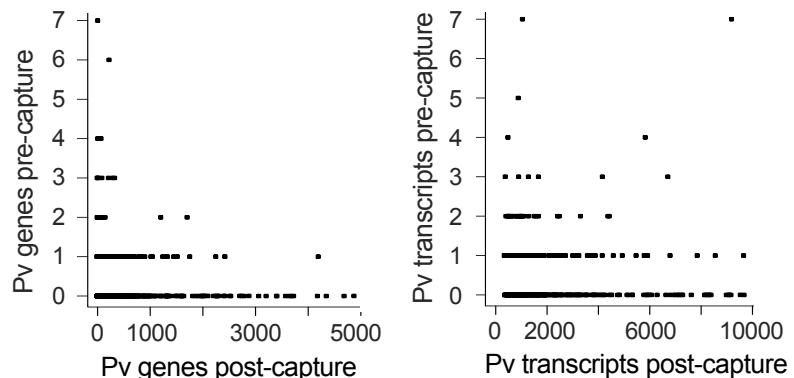
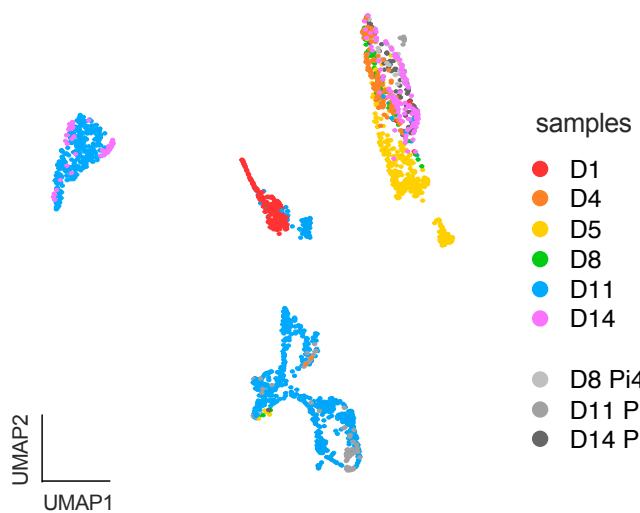
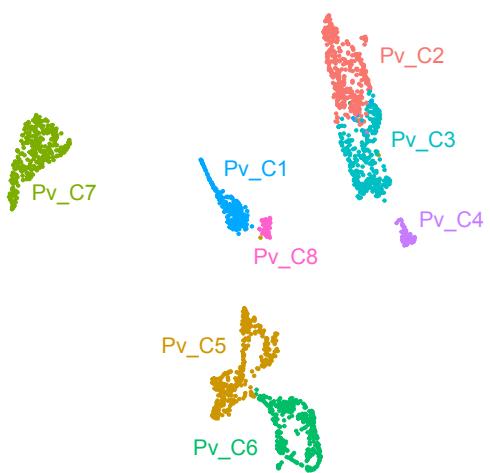
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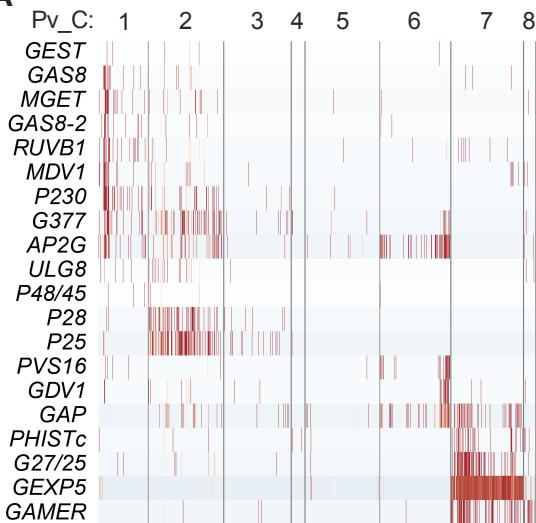
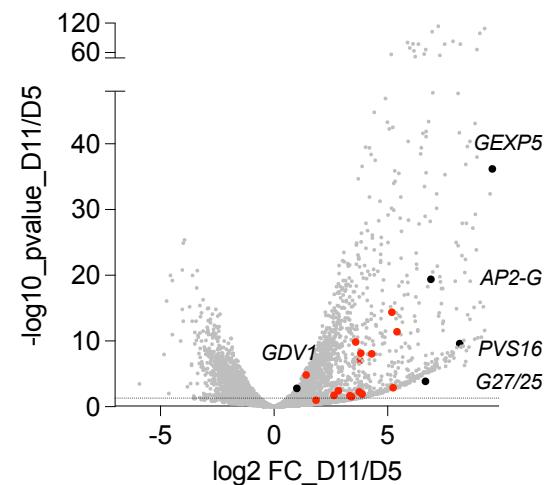
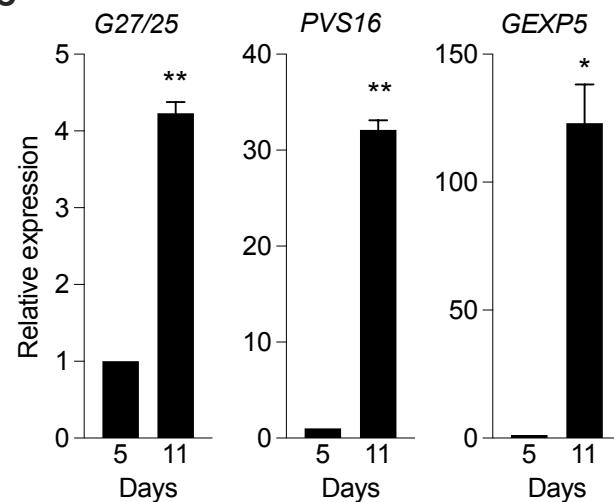
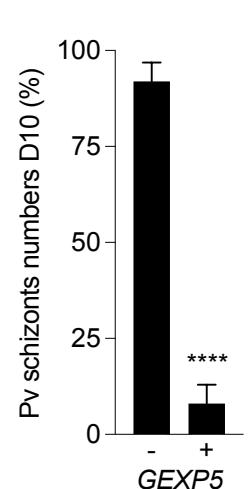
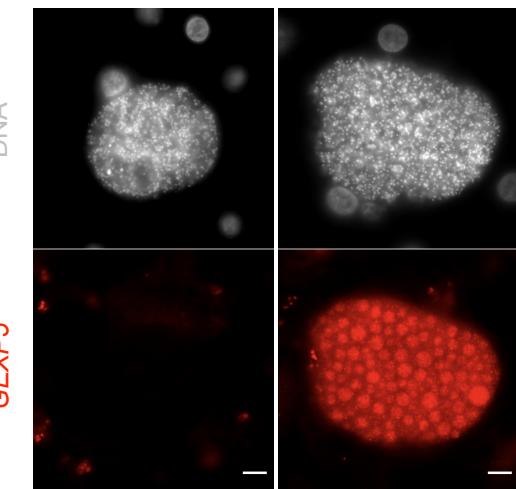
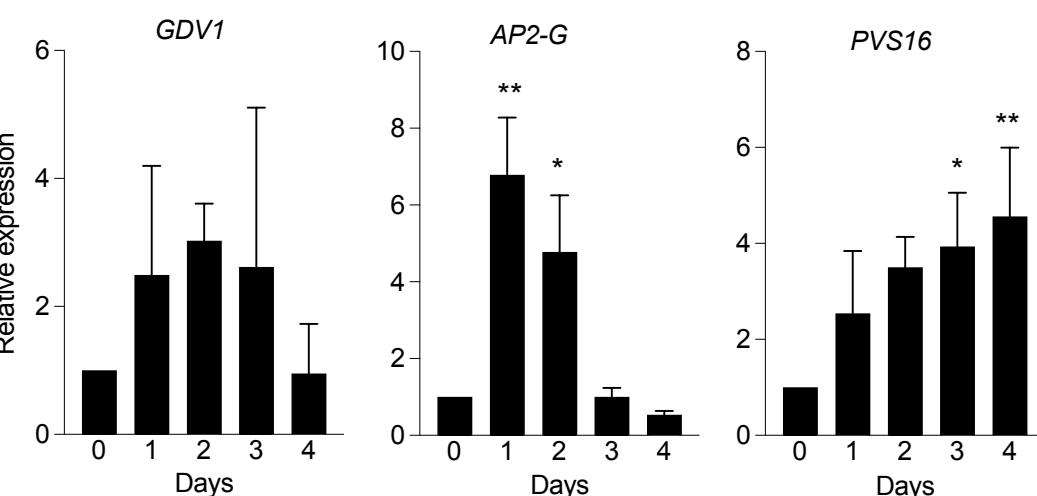
641

642 Walzer, K.A., Kubicki, D.M., Tang, X., and Chi, J.-T.A. (2018). Single-Cell Analysis Reveals Distinct  
643 Gene Expression and Heterogeneity in Male and Female *Plasmodium falciparum* Gametocytes . *MSphere*  
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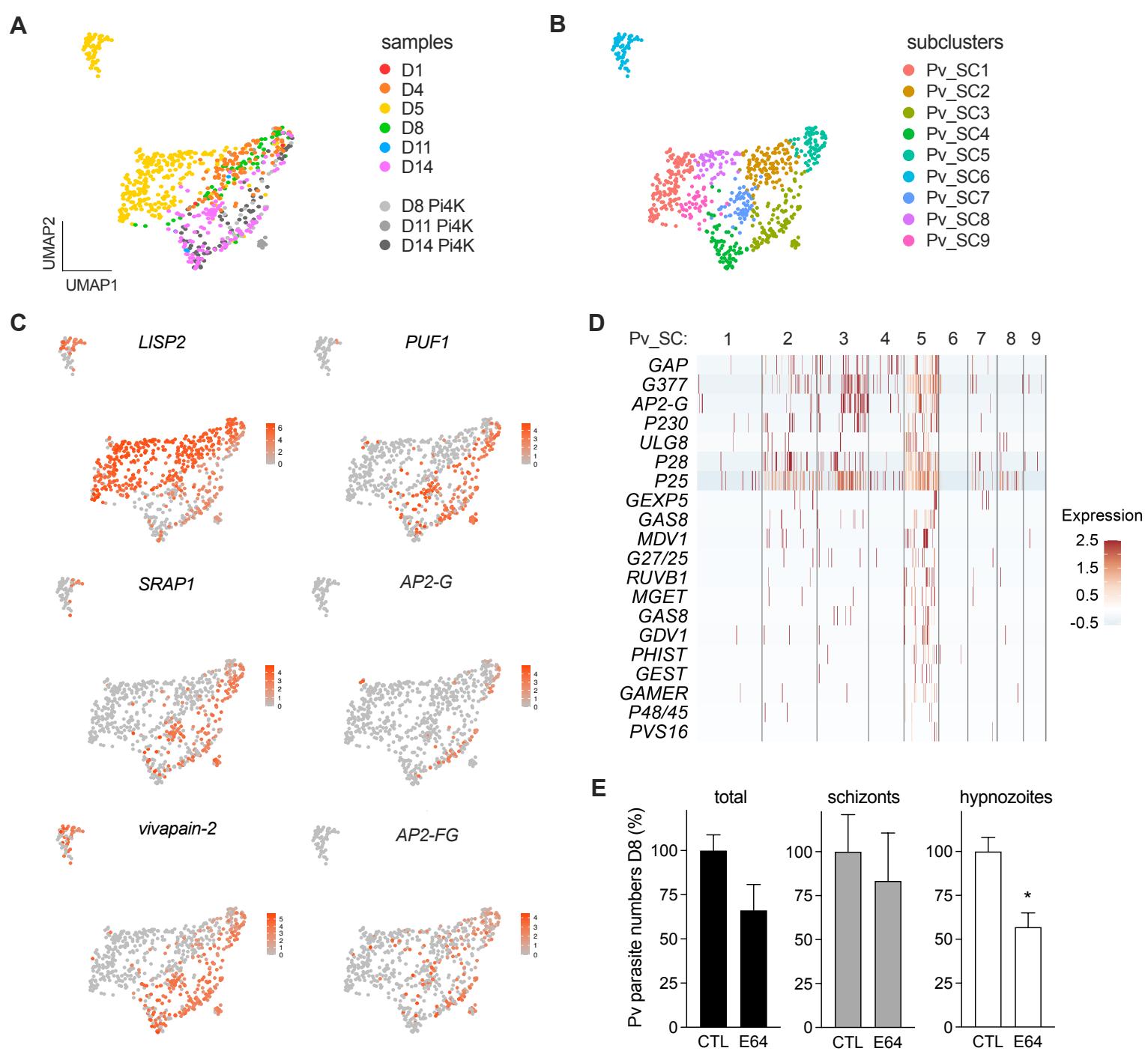
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**A****B****C****D****E**

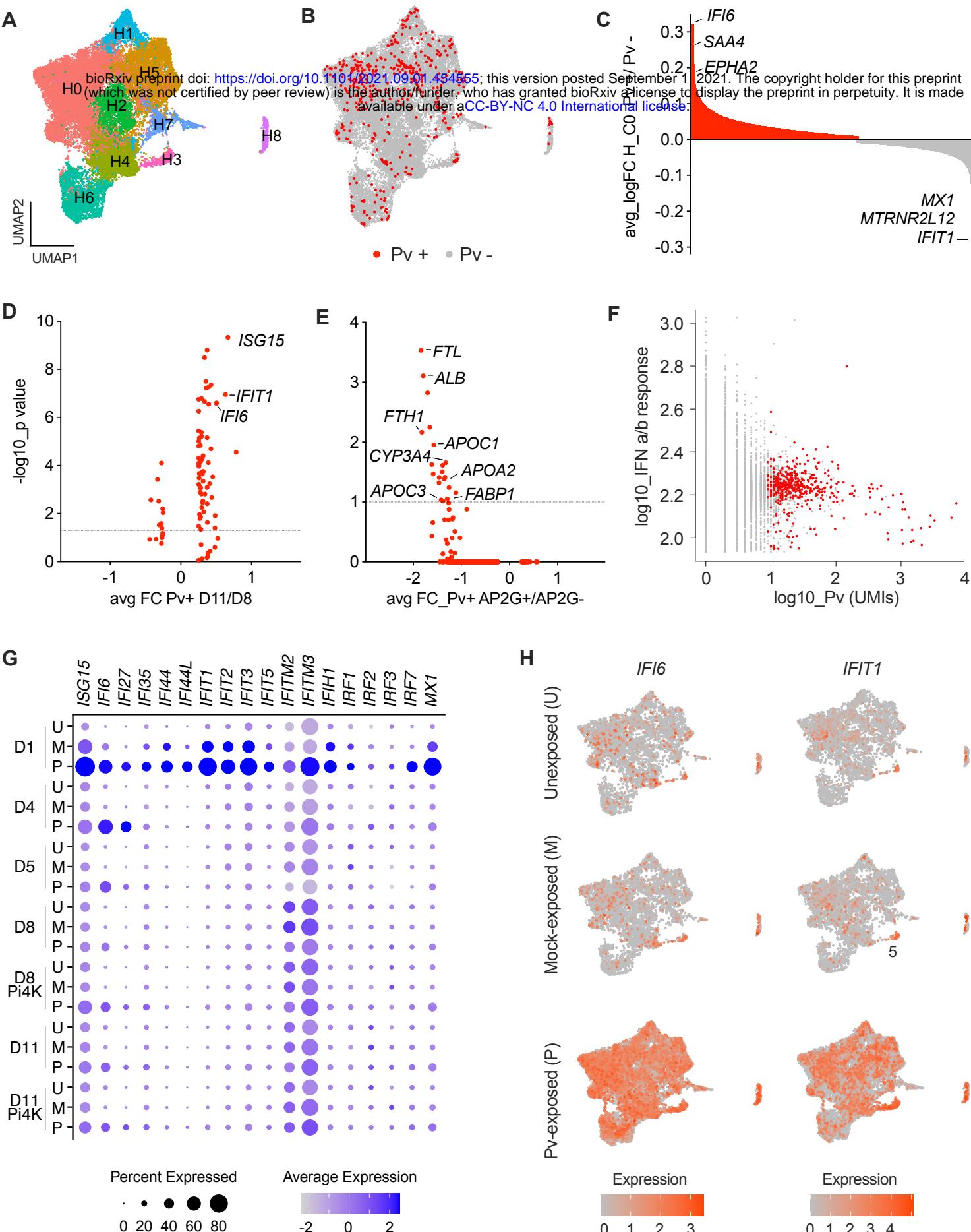
**Figure 1.** Single-cell profiling of *P. vivax* liver-stage infection. **A.** Illustration of *P. vivax* liver-stages (purple) maintained in micropatterned co-cultures (MPCC) of human hepatocytes (brown) and supportive mouse fibroblasts (blue). Cultures were dissociated with trypsin (top) or directly collected in bulk (bottom). Sample processing pipeline is shown for whole transcriptome (top) and targeted (middle) single-cell and targeted bulk RNA sequencing (bottom). **B.** Parasite size distribution and collection days. Day 14 and Pi4K-treated samples are enriched in nonreplicative hypnozoites (parasites  $< 10 \mu\text{m}$  diameter). Each dot represents an individual parasite ( $n = 3-8$  wells pooled from 2 independent infections). Quantification of parasite numbers given in **Figure S1A**. **C.** Scatterplots comparing the number of *P. vivax* genes (left) and transcripts (right) prior and post-capture with parasite-specific baits. Efficient depletion of host genes shown in **Figure S1B**. **D-E.** UMAP of 1,991 individual *P. vivax* parasites colored by sample identity (**D**) and parasite cluster type (**E**) using Scanorama. Heatmap showing cluster-specific marker genes provided in **Figure S2A**.

**A****B****C****D****E**

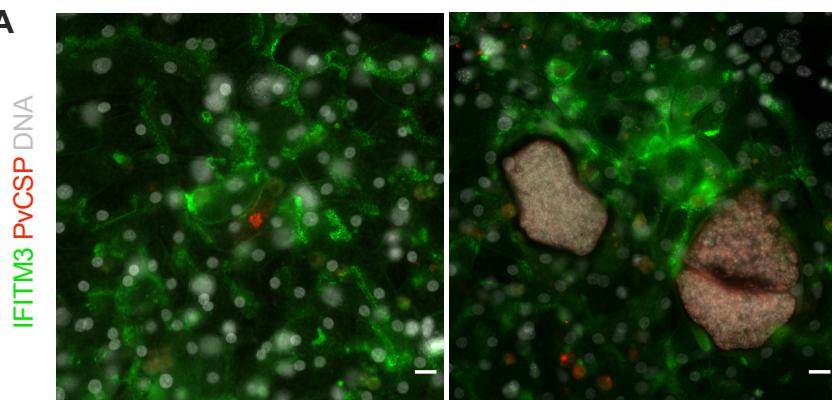
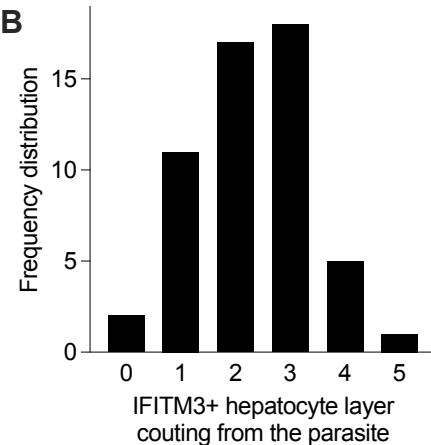
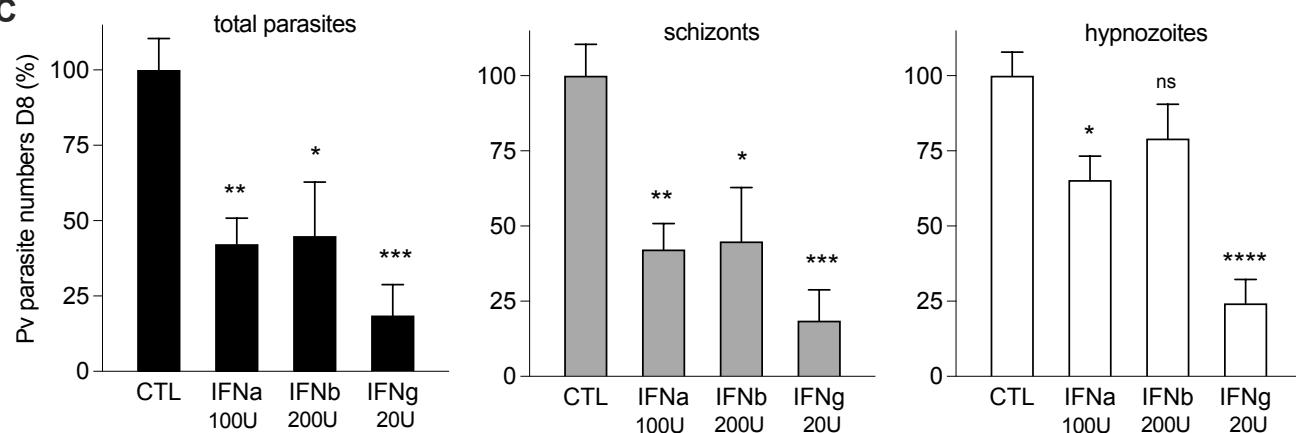
**Figure 2.** Characterization of *P. vivax* individual transcriptomes. **A.** Heatmap showing the expression of gametocyte-specific genes for each parasite cluster. 100 parasites are shown, except clusters Pv\_C4 and Pv\_C8 that contain fewer cells. Each bar represents a single parasite. **B.** Volcano plot comparing differential expression of day 5 and day 11 samples on targeted bulk RNA-seq. Parasite gametocyte gene markers highlighted. Red for gametocyte genes described in (Sà et al., 2020). Black for genes validated in **C-E**. **C.** Relative expression of gametocyte markers by quantitative RT-PCR (mean  $\pm$  SEM;  $n = 2$  independent infections;  $t$ -test: \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ). **D.** RNA in situ hybridization of *P. vivax* parasites at day 10. *GEXP5* transcripts shown in red. Scale bars, 10  $\mu$ m. Quantification of *GEXP5*-positive and -negative schizonts (mean  $\pm$  SEM;  $n = 4$  wells;  $t$ -test: \*\*\*\*,  $p < 0.0001$ ). **E.** Relative expression of *GDV1*, *AP2-G* and *PVS16* by quantitative RT-PCR at early timepoints (mean  $\pm$  SEM;  $n = 2$  independent infections; 1-way ANOVA test: \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ). FC, fold change; *G27/25*, gametocyte antigen; *PVS16*, parasitophorous vacuole membrane protein S16; *GAMER*, gamete release protein; *GEXP5*, gametocyte exported protein 5; *GDV1*, gametocyte development 1; *AP2-G*, AP2 domain transcription factor regulator of gametocytogenesis.



**Figure 3.** Characterization of *P. vivax* mid-stage transcriptomes. **A-B.** UMAP of 713 *P. vivax* parasites (Pv\_C2-C4 re-clustered) colored by sample identity (**A**) and parasite subcluster types (**B**). **C.** UMAP as in **A** highlighting expression levels of *LISP2*, *PUF1*, genes encoding peptidases (*SRAP1* and *PVP01\_0916200* *vivapain-2*) and *ApiAP2s* (*AP2-G* and *AP2-FG*). **D.** Heatmap showing expression of gametocyte-specific genes for each parasite subcluster. Gene list as in **Figure 2A**. Each bar represents a single parasite. Expression of gametocyte markers in Pi4K-treated samples is shown in **Figure S3A**. **E.** Treatment (days 0-8) of *P. vivax* infected cultures with protease inhibitor E64 (1  $\mu$ M; mean  $\pm$  SEM;  $n = 3-5$  wells;  $t$ -test: \*,  $p < 0.05$ ). Parasite size distribution is given in **Figure S3B**. *LISP2*, liver-specific protein 2; *PUF1*, Pumilio RNA binding protein; *SRAP1*, sea star regeneration associated protease; *AP2-G*, transcription factor regulator of gametocytogenesis; *AP2-FG*, female gametocyte-specific transcriptional regulator.



**Figure 4.** Dual scRNA-seq analysis of *P. vivax* liver-stage infection. **A.** UMAP of 31,767 individual cells colored by cell type cluster using Seurat. Sample identity and cell composition within each human cluster are shown in **Figures S4A-B**. **B.** UMAP as in **A** highlighting the *P. vivax*-positive hepatocytes in red. GSEA analysis in **Figure S4C**. **C.** Waterfall plot comparing *P. vivax*-positive versus -negative in cluster H0 (adjusted  $p < 0.05$ ). **D-E.** Volcano plots comparing differential expression of *P. vivax*-positive cells on day 11 and day 8 (**D**) and AP2-G positive and negative (**E**). **F.** Scatter plot showing higher interferon (IFN) response in *P. vivax*-negative cells (grey) across all clusters. *P. vivax*-positive hepatocytes are colored in red in **C-F**. **G.** Dot plot showing expression of IFN-related genes throughout infection time course in naïve unexposed (U), mock-exposed (M) and *P. vivax*-exposed (P) samples. **H.** UMAP as in **A** highlighting *IFI6* and *IFIT1* expression levels in U, M and P samples.

**A****B****C**

**Figure 5.** IFN responses in *P. vivax*-infected and bystander hepatocytes. **A.** Expression of IFITM3 protein (green) in *P. vivax*-infected cultures at day 10. Representative images of small (left) and large (right) parasites. Scale bars, 20  $\mu$ m. **B.** Quantification of IFITM3 positive hepatocytes relative to the infected cell. **C.** IFN treatment (days 5-8) of *P. vivax* infected cultures. Bar plots show quantification of parasite numbers at day 8 (mean  $\pm$  SEM;  $n = 5$ -6 wells pooled from 2 independent infections; 1-way ANOVA test: \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ ; \*\*\*\*,  $p < 0.0001$ ; ns, not significant). Parasite size distribution is given in **Figure S5**.