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1 Antibody-independent mechanisms regulate the establishment of
2 chronic *Plasmodium* infection

3

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24

25 Malaria is caused by parasites of the genus *Plasmodium*. All human-infecting
26 *Plasmodium* species can establish long-lasting chronic infections¹⁻⁵, creating an
27 infectious reservoir to sustain transmission^{1,6}. It is widely accepted that maintenance
28 of chronic infection involves evasion of adaptive immunity by antigenic variation⁷.
29 However, genes involved in this process have been identified in only two of five
30 human-infecting species: *P. falciparum* and *P. knowlesi*. Furthermore, little is
31 understood about the early events in establishment of chronic infection in these
32 species. Using a rodent model we demonstrate that only a minority of parasites from
33 among the infecting population, expressing one of several clusters of virulence-
34 associated *pir* genes, establishes a chronic infection. This process occurs in different
35 species of parasite and in different hosts. Establishment of chronicity is independent
36 of adaptive immunity and therefore different from the mechanism proposed for
37 maintenance of chronic *P. falciparum* infections⁷⁻⁹. Furthermore, we show that the
38 proportions of parasites expressing different types of *pir* genes regulate the time
39 taken to establish a chronic infection. Since *pir* genes are common to most, if not all,
40 species of *Plasmodium*¹⁰, this process may be a common way of regulating the
41 establishment of chronic infections.

42

43 Long-lasting *Plasmodium falciparum* infections are thought to be maintained by
44 cytoadherence to avoid splenic clearance and antigenic variation of the adherent proteins
45 to avoid clearance by antibodies. We wanted to know how chronic infections are
46 established by malaria parasites lacking genes known to be involved in these processes
47 (*var* and *sicavar*). We used, as an example, the rodent malaria parasite *Plasmodium*
48 *chabaudi chabaudi* AS that gives rise to chronic infections in laboratory mice (Figure
49 1A). The acute infection is defined by a peak of parasitaemia (5-10% iRBC)
50 approximately ten days after mosquito bite, and clearance of the majority of parasites by
51 day fifteen. A chronic infection is then established, giving rise to several episodes of
52 patent parasitemia (>0.01%) for up to eighty days. Comparison of the transcriptomes of
53 parasite populations from the acute and chronic phases of infection showed that among
54 the multigene families identified in the *P. chabaudi* AS genome, the *pir* family is the
55 most differentially expressed (Figure 1A, Supplementary Tables 1 and 2). This multigene
56 family is present in most *Plasmodium* genomes¹¹ and has been associated with
57 virulence¹². More than half of the *pir* genes were differentially expressed between acute
58 and chronic phases. The majority of these genes had significantly higher expression in the
59 acute phase of infection, whereas only a few had higher expression in the chronic phase.

60 A *Plasmodium*-specific antibody response is detectable from day seven of
61 infection¹³ and thus the change in *pir* expression might be the result of adaptive
62 immunity-dependent selection of parasites expressing certain *pir* genes. However, we
63 observed the same change in mice lacking B cells and antibodies (μ MT; Figure 1B) or
64 lacking T cells able to promote an antibody response (TCR α -/-; Figure 1C;

65 Supplementary Figure 1; Supplementary Table 1). Therefore the transcriptomic changes
66 observed between acute and chronic phases are independent of adaptive immunity and
67 distinct from the mechanism suggested to be involved in maintenance of chronic
68 infection in *P. falciparum*⁹.

69 In the genomes of most malaria parasites, *pir* genes are located in subtelomeric
70 regions that are difficult to resolve based on the *de novo* assembly of short sequencing
71 reads. Using Single Molecule Real-Time (SMRT) sequencing, we generated a new
72 genome assembly for *P. chabaudi* AS comprising a complete set of fourteen
73 chromosomes with no gaps (Supplementary Table 3). We observed that some
74 subtelomeres had similar sets of *pir* genes arranged in clusters (Supplementary Figures 2
75 and 3). In recent work, *pir* genes were classified into several short and long forms (S1-7
76 and L1-4, respectively)¹⁴. We found eight clusters with common structures: three were
77 enriched for the L1 subfamily and five were enriched for the S7 subfamily (Figure 2;
78 Supplementary Table 4). Interestingly, we found that the majority of *pir* expression in our
79 samples came from this small number of clusters, and that genes within clusters were co-
80 expressed during infection (Supplementary Figure 4). While the S7-rich clusters were
81 highly expressed during the acute phase, the chronic phase was dominated by expression
82 of L1-rich loci (Figure 2C; Supplementary Table 5). This pattern was replicated in the
83 $\alpha\beta$ T-cell receptor- and B-cell knockout mice (Figure 2C) reinforcing our earlier
84 conclusion that changes in *pir* gene expression are independent of selection by the
85 adaptive immune response. Individual L1- or S7-rich loci are hereafter termed
86 *Chronicity-Associated Pir Locus* (ChAPL), and *Acute-Associated Pir Locus* (AAPL),
87 respectively. We identified an intergenic motif, commonly occurring upstream of most

88 ChAPL genes, that might serve as a promoter sequence (Supplementary Figure 5).
89 However, as only a subgroup of ChAPL genes are expressed in each mouse (Figure 2C),
90 it is likely that epigenetic mechanisms regulate access of transcription factors to this
91 motif, as shown for subtelomeric gene families in *P. falciparum*¹⁵. Of the three ChAPLs,
92 we observed a preference for clusters on chromosome 6.

93 We recently showed that increased *P. chabaudi* virulence during serial blood
94 passage (SBP) is associated with a reduced repertoire of *pir* gene transcripts compared
95 with parasites transmitted by mosquito (MT)¹². The gene most highly upregulated in SBP
96 parasites was a *pir* from the L1 subfamily (PCHAS_1100300). Although not found in a
97 ChAPL, this gene is from the same subfamily that is most common in these loci. We
98 therefore hypothesized that chronic phase parasites expressing ChAPLs would have a
99 similar phenotype. To test this, we injected acute and chronic phase parasites into naïve
100 mice. Chronic parasites gave rise to significantly greater parasitaemias and pathology
101 than parasites derived from the acute phase of infection (Figure 3A; Supplementary
102 Figure 6A). This suggests that parasite populations derived from the chronic phase
103 expand better in the face of the response of a naïve mouse than parasites derived from the
104 acute phase. However, as chronic parasites only reached high parasitaemias when
105 injected into naïve mice, our data also indicate that they are better controlled when the
106 host has previously experienced an acute infection. This pattern was also observed when
107 chronic parasites were collected from B-cell knockout mice (Supplementary Figure 6B).
108 Chronic infections therefore involve the emergence of phenotypically distinct parasites
109 expressing ChAPLs, independently of adaptive immunity.

110 Thus we have established that there are transcriptional and phenotypic changes
111 observed during chronic *Plasmodium* infections and that they do not stem from classical
112 antigenic variation. We then sought to determine whether these were due to a
113 transcriptional switch at the population level, or to a selection of pre-existing parasites
114 from the acute phase. We isolated single parasites from the acute and chronic phases of
115 infection, initiated clonal infections in C57Bl/6 mice and performed RNA sequencing
116 analyses of these cloned populations of parasites (Figure 3B). We found that one out of
117 ten acute-phase clones was more virulent than the population as a whole (Figure 3B;
118 Supplementary Figure 7). While avirulent clones expressed AAPLs, the virulent clone
119 expressed a ChAPL, as predicted by our finding that ChAPL-expressing parasites are
120 more virulent in naïve mice (Figure 3C; Supplementary Figure 8; Supplementary Table
121 6). As expected, the clones from the chronic phase were all virulent and expressed
122 ChAPLs (Figures 3B & 3C, Supplementary Figures 8 & 9). This suggests that the
123 establishment of chronic infection involves selection of a small proportion of parasites
124 expressing ChAPLs from a clonally variant population largely expressing AAPLs.

125 On the basis of these observations, we hypothesized that the proportion of virulent
126 parasites already in the acute phase might affect the success of establishing a chronic
127 infection. In our initial transcriptome experiment, we found that high expression of
128 ChAPLs during the acute phase was associated with a more rapid recrudescence
129 (Supplementary Table 7). We then tested whether chronic-like parasites, expressing L1
130 *pir* genes, outgrew acute parasites largely expressing AAPLs. We used SBP parasites as a
131 proxy for chronic parasites as they express the L1 *pir* PCHAS_1100300, and have a
132 similar phenotype to ChAPL-expressing chronic parasites. We performed mixed

133 infections in C57BL/6 mice with different ratios of SBP and acute MT (expressing
134 AAPLs) parasites tagged with different fluorescent markers (Figure 4, Supplementary
135 Figure 10 A-C; Supplementary Table 8). SBP parasites rapidly outcompeted MT
136 parasites (Figure 4; Supplementary Figure 10D), confirming that the more virulent,
137 chronic-like parasites had a selective advantage, as has been shown previously for
138 different parasite isolates¹⁶⁻²⁰. The selection of a minority of iRBCs, expressing
139 virulence-associated *pir* genes (Figures 3B & 3C), can therefore explain the change in
140 expression from S- to L-*pir* genes observed in the parasite population between the acute
141 and chronic phases of infection (Figures 1 & 2). As we had hypothesized, higher doses
142 of L1-expressing parasites resulted in an earlier recrudescence confirming that these
143 parasites have a better capacity to establish a chronic infection (Figure 4, Supplementary
144 Figure 11).

145 When a similar infection mixing SBP and MT parasites was carried out in RAG1^{-/-} mice
146 (lacking both T and B cells) and RAG^{-/-} mice treated with chlodronate liposomes to
147 deplete macrophages (Supplementary Figure 12A), virulent parasites similarly rapidly
148 outgrew AAPL-expressing parasites. Thus, this early selection takes place even in the
149 absence of B cells, T cells, and macrophages indicating that other mechanisms are
150 operating (Supplementary Figure 12B).

151 Unexpectedly, when mice were infected with 10⁴ virulent parasites plus 10⁵
152 avirulent parasites, the acute parasitaemia and pathology were lower than in mice
153 infected with 10⁴ virulent parasites alone (Figure 4B, Supplementary Figure 11). This
154 suggests that AAPL-expressing parasites act to lower the virulence of parasites
155 expressing largely virulence-associated L1 *pirs* and delay the onset of chronicity.

156 Therefore, the initial composition of parasites expressing different *pir* genes regulates
157 parasite recrudescence.

158 To determine whether our findings apply more generally, beyond *P. chabaudi* AS
159 infections of laboratory mice, we established chronic infections with alternative
160 host/parasite combinations. We used the more virulent *P. chabaudi* CB strain in C57BL/6
161 mice and *P. chabaudi* AS in the African thicket rat *Grammomys surdaster*. This is a
162 natural host of rodent *Plasmodium* (Supplementary Figures 13A & 13B). Reduced
163 AAPL and increased ChAPL gene expression during chronic *P. chabaudi* infection were
164 observed in both cases, suggesting that our findings are a general feature of *P. chabaudi*
165 chronic infections irrespective of strain of parasite or host (Supplementary Figures 13C &
166 13D). We then investigated chronic *P. berghei* infections in Brown Norway rats
167 (Supplementary Figure 14A). The subtelomeric regions and *pir* repertoire of *P. berghei*
168 are quite different from those of *P. chabaudi*¹⁴. Loci resembling the AAPLs and ChAPLs
169 of *P. chabaudi* are absent. Nevertheless, we observed a general decrease in *pir* expression
170 during the chronic phase with an increase in expression of L2 *pir* genes (Supplementary
171 Table 9; Supplementary Figure 14B). The L2 *pir* genes of *P. berghei* are similar to the *P.*
172 *chabaudi* L1 genes we found in ChAPLs. They are present in recently copied loci
173 (Supplementary Figure 14C), contain long variable central domains and have a longer N-
174 terminal domain that may contain a second transmembrane helix (Supplementary Figure
175 15). This suggests our findings are a general feature of malaria parasites infecting rodents.

176 Our results show that mosquito transmission of *P. chabaudi* gives rise to a
177 clonally variant population of parasites, the majority expressing AAPLs and a minority
178 ChAPLs. During the acute phase of infection, AAPL-expressing parasites are controlled

179 by the host response, while ChAPL-expressing parasites survive and establish a chronic
180 infection. The more ChAPL-expressing parasites that are present early in infection, the
181 more rapidly the chronic infection is established.

182

183 While it is thought that maintenance of chronic *P. falciparum* infection depends
184 on the differential expression of *var* genes^{7,8}, how similar infections are established and
185 maintained by *P. vivax*, *P. malariae* and *P. ovale*, that lack these variant antigens, is
186 unknown. Here, we show that the establishment of chronic *Plasmodium* infection in
187 rodent models is reproducibly associated with phenotypically distinct parasites,
188 expressing particular *pir* gene clusters. Importantly, contrary to what is understood about
189 maintenance of chronic infection in *P. falciparum*⁹, this change in the parasite population
190 is independent of adaptive immunity. Antigenic variation may still operate in maintaining
191 chronicity, but our results clearly show that other mechanisms allow for the establishment
192 of chronic infection in parasites that infect rodents.

193 Our finding that the initial population of parasites comprises individuals, each
194 expressing different *pir* genes, suggests that the chronic infection is established by a
195 small number of parasites which evade the early immune response. The selective
196 advantage during infection of ChAPL-expressing over AAPL-expressing parasites could
197 be of multiple origins. Although our previous results showed that growth rates of
198 avirulent MT parasites and virulent SBP parasite were similar in RAG1^{-/-} mice¹², red cell
199 invasion efficiency might still play a role in selection of virulent clones for establishment
200 of chronic infection. We are also investigating rosette formation^{21,22}, red cell
201 deformability²³, sequestration²⁴, and the capacity to escape a macrophage-independent

202 innate immune response²⁵ as possibilities. It has been shown that the expression of
203 subsets of *P. falciparum* *rif* genes (which may be relatives of *pir* genes¹⁰) and *P. vivax* *pir*
204 genes are associated with cytoadherence of iRBCs to human endothelial cells^{26,27} and red
205 blood cells^{21,28,29}, two processes implicated in parasite survival and pathogenicity³⁰.
206 Furthermore, it has previously been suggested that the same molecules are involved in
207 cytoadherence and immune evasion in *P. chabaudi*^{9,24}. Differences in cytoadhesive
208 properties could therefore promote the expansion of more virulent parasites.
209 Characterizing this process and determining the function of *pir* genes *in vivo* may identify
210 mechanisms that universally regulate immune evasion by *Plasmodium* and lead to novel
211 approaches to reduce their survival and transmission.

212 **Methods**

213

214 **Mouse and parasite lines used**

215 All experiments were performed in accordance with the UK Animals (Scientific
216 Procedures) Act 1986 (PPL 80/2538) and the guidelines provided by the
217 National Institutes of Health (NIH) Animal Care and Use Committee (ACUC) and
218 approved by the Francis Crick Institute Ethical Committee and by the Institutional
219 Animal Care and Use Committee (IACUC). T cell receptor- α chain knockout (TCR α -/
220)³¹, mice homozygous for a targeted mutation of the transmembrane exon of the IgM μ
221 chain, μ MT³², and V(D)J recombination activation gene RAG-1 knockout (RAG-1-/-)³³
222 on a C57Bl/6 background, and C57 Bl/6 Wildtype (WT) mice were obtained from the
223 specific-pathogen free (SPF) unit and subsequently conventionally housed with
224 autoclaved cages, bedding and food at the BRF, of the Francis Crick Institute. The
225 *Grammomys surdaster* rats used in this study were captured in the wild at Fungurume and
226 Lumata in the Katanga province, south of DR Congo, were bred and maintained in a
227 conventional unit in sterile cages, with autoclaved water and bedding at
228 NIH/NIAID/LMIV³⁴. Inbred Brown Norway rats were obtained from Charles River
229 Laboratories and housed at the Animal Holding Unit of the Wellcome Trust Sanger
230 Institute. Experiments were performed with 6-8 week old female mice and *Grammomys*
231 *surdaster* rats housed under reverse light conditions (light 19:00-07:00, dark 07:00-
232 19:00) and 6-week old female Brown Norway rats housed under normal light conditions,
233 at 20-22°C. Measurements of clinical pathology were taken at 11:00. Core body
234 temperature was measured with a rectal thermometer and erythrocyte density was
235 determined with a VetScan HMII haematology system (Abaxis). Changes in body

236 temperature, body weight and erythrocyte density were calculated relative to a baseline
237 measurement performed 1 day before infection. An infection was considered virulent
238 when it induced a statistically significant decrease in the mouse temperature, weight
239 and/or red blood cells compared with uninfected control mice.

240 Cloned lines of *Plasmodium chabaudi chabaudi* AS and CB parasites were used. To
241 initiate infection, mice were either injected intraperitoneally (IP) or intravenously (IV)
242 with 1×10^4 or 10^5 infected erythrocytes or submitted to the bite of 20 infected mosquitoes
243 as previously described¹². *Grammomys surdaster* rats were infected by I.V. injection of
244 2500 sporozoites of *Plasmodium chabaudi chabaudi* AS. Brown Norway rats were
245 infected by I.V. injection of 7200 sporozoites of *Plasmodium berghei* ANKA.
246 Parasitaemia was monitored by Giemsa-stained thin blood films on blinded samples. The
247 limit of detection for patent parasitaemia was 0.001% infected erythrocytes.

248

249 **Chronic infections in wild type and genetically altered rodents**

250 Out of 10 wild-type C57Bl/6 mice, 10 μ MT mice and 10 TCR α ^{-/-} mice infected with *P.*
251 *chabaudi* AS via mosquito bite, 9 WT C57Bl/6 mice, 5 μ MT mice and 3 TCR α ^{-/-} mice
252 were randomly selected for transcriptomic analysis. 3 C57Bl/6 mice infected with *P.*
253 *chabaudi* CB, 3 *Grammomys surdaster* rats infected with *P. chabaudi* AS and 3 Brown
254 Norway rats infected with *P. berghei* ANKA (all via mosquito bites or injection of
255 sporozoites) were also used for transcriptomic analysis. Our aim was to use a minimum
256 of three biological replicates per condition for all experiments, as this is considered to be
257 sufficient for general surveys of differential expression³⁵. Blood was extracted from the
258 same rodents at two time points, therefore analysing the changes occurring over time in

259 the same parasite populations. During the acute phase of infection, 100 μ L of blood was
260 collected from the tail after the completion of seven cycles of schizogony. During the
261 chronic phase, exsanguination was performed between 30 and 40 days post-infection in
262 μ MT mice, at 30 days post-infection in TCR α ^{-/-} mice, at day 15 post-infection in Brown
263 Norway rats or when the parasitaemia reached 0.1% between 27 and 40 days post-
264 infection in wild type C57Bl/6 mice and *G. surdaster* rats. After data collection, the acute
265 sample from wild-type mouse 454 and both acute and chronic samples from μ MT mouse
266 433 were excluded, as they were outliers in a multi-dimensional scaling analysis of the
267 gene expression data.

268

269 **Phenotypic analysis of acute and chronic parasites**

270 Donor mice were infected via mosquito bites. Parasites from donor mice ($n= 3$) were
271 blood-passaged into recipient mice at 1, 3, 4 and 6 weeks post-mosquito transmission.
272 Recipient mice ($n=5$ per donor mouse, the minimum number required for an 80% chance
273 of significant differences ($p\leq 0.05$) in parasitemia, weight and erythrocyte counts) were
274 infected by IP injection of 10^5 infected erythrocytes. Parasitaemia and signs of pathology
275 were monitored in recipient mice on blinded samples.

276

277 **Cloning of single parasites from acute and chronic phases**

278 One mouse was infected via mosquito bite. Infected blood was then collected from the
279 tail at two time points: 1 week post-infection (acute phase) and six weeks post-infection
280 (chronic phase). Both times, parasites were cloned by dilution and single parasites were
281 injected IV in wild type mice. After expansion, each cloned line was blood passaged into

282 eight experimental mice by IP injection of 10^5 infected erythrocytes. Five mice were used
283 to monitor parasitaemia and signs of pathology and three mice were sacrificed and
284 exsanguinated after the completion of seven cycles of schizogony for RNA extraction.

285

286 **RNA extraction**

287 During all RNA extractions experiments, blood-stage parasites were isolated at 11:00,
288 when >90% of the parasites were at the late trophozoite stage of development (Table S8).
289 Parasite RNA was isolated as previously described¹². Briefly, whole blood was depleted
290 of leukocytes by filtration (Plasmodipur, EuroProxima) and from globin RNA and red
291 cell debris by saponin lysis (Sigma) and centrifugation. Purified parasites were
292 resuspended in TRIzol, snap-frozen on dry ice and kept at -80°C until use. RNA was then
293 extracted, resuspended in water and its quantity and quality were determined on an
294 Agilent 2100 Bionalayzer RNA 6000 Nano or Pico chip.

295

296 **RNA-seq library prep and sequencing**

297 The majority of RNA was used to make 200-450bp fragment libraries using Illumina's
298 TruSeq RNA Sample Prep v2 kit, with 10 cycles of PCR amplification using KAPA Hifi
299 Polymerase rather than the kit-supplied Illumina PCR Polymerase. Two samples required
300 14 cycles of PCR (T1_46-4 and T1_47-4). Mosquito-transmitted *P. chabaudi* CB
301 samples (MTCB) were subjected to 15 cycles of PCR.

302 The libraries were sequenced using an Illumina HiSeq 2000 v3 with 100bp paired-end
303 reads (samples beginning 'T'), an Illumina HiSeq 4000 with 75bp paired-end reads
304 (MTCB samples) or an Illumina HiSeq 2500 with 75bp paired-end reads for the rest.

305 RNA from cloned parasites was used to make 100-300bp fragment libraries produced
306 using Illumina's TruSeq Stranded mRNA Sample Prep Kit with 10 cycles of PCR
307 amplification using KAPA Hifi Polymerase. These libraries were sequenced using an
308 Illumina HiSeq 2500 with 75bp paired-end reads. A description of the libraries can be
309 found in Supplementary Table 10.

310

311

312 **Analysis of gene expression**

313 Tag sequences were removed from sequenced reads. Where individual samples were run
314 over multiple lanes, the reads from these were merged before mapping. Reads were then
315 mapped against spliced gene sequences (exons, but not UTRs) from the v3 *P. c. chabaudi*
316 AS (this work) or the v3 *P. berghei* ANKA (Fougère et al., under review) reference
317 genomes using Bowtie2 v2.1.0³⁶ (-a -X 800 -x). Read counts per transcript were
318 estimated using eXpress v1.3.0³⁷, with default parameters. Genes with an effective
319 length cutoff below 10 in any sample were removed. Summing over transcripts generated
320 read counts per gene.

321 Differential expression analysis was performed using edgeR v3.8.6³⁸ on genes with ≥ 3
322 counts per million. The Fisher's exact test was used with cutoffs FDR < 0.01 and fold
323 change ≥ 2 , except for the *P.berghei* analysis where FDR < 0.1 and fold change ≥ 1 was
324 used. Functional categories of genes were identified by orthology using GeneDB³⁹ from
325 several different *P. falciparum* datasets: invasion genes⁴⁰, sexual genes⁴¹ and
326 subtelomeric (by manual inspection of chromosomes).

327 Gene expression clustering was used to identify which genes within ChAPLs and AAPLs
328 were co-expressed. This was done by generating a toroidal 5x5 Self-Organising Map
329 (SOM) using the Kohonen package in R⁴². FPKM values for each gene were logged (base
330 2) and mean-normalised per gene. The SOM was trained in 100 iterations.

331

332 **Sequencing and assembly of *P. c. chabaudi* AS v3 genome**

333 High molecular weight DNA was prepared as follows. Heparinised blood from two mice
334 infected with *P. chabaudi* AS (blood passaged parasites, days seven & eight post-
335 infection) was pooled, diluted 1:3 in PBS, and passed through a Plasmodipur (Euro-
336 Diagnostica) filter. Erythrocyte membranes were removed by saponin lysis (0.15% in ice
337 cold PBS). Parasitised erythrocytes were recovered by centrifugation (2000g 10 min
338 4 °C) and washed twice in ice cold PBS. The cell pellet was taken up in 50mM Tris HCl
339 pH 7.5, 50mM EDTA pH8.0, 100mM NaCl, 0.5% SDS and digested with RNase A
340 (1mg/ml, Life Technologies) for 30 min at 37°C. Proteinase K (Roche) was added to a
341 final concentration of 1mg/ml, incubated at 45°C overnight, followed by extraction with
342 phenol chloroform and ethanol precipitation.

343 For preparation of long-read sequencing libraries, 5µg of *P. c. chabaudi* AS genomic
344 DNA was sheared to 20-25kb by passing through a 25mm blunt ended needle. SMRT
345 bell template libraries were generated using the Pacific Biosciences issued protocol (20
346 kb Template Preparation Using BluePippin™ Size-Selection System). After 7kb-20kb
347 size-selection using the BluePippin™ Size-Selection System (Sage Science,
348 Beverly, MA) the library was sequenced using P6 polymerase and chemistry version 4
349 (P6C4) on 5 single-molecule real-time (SMRT) cells, each with a 240 minutes movie

350 length. The five SMRT cells were processed using a Pacific Biosciences RSII. Reads
351 were filtered using SMRT portal v2.2 with default parameters (minimum subread length
352 50, minimum polymerase read quality 75, minimum polymerase read length 50). The
353 yield was 258214 reads totaling 1.51Gb, with read N50 9003bp and read quality 0.833.
354 The reads were assembled using HGAP v2.2.0⁴³ with expected coverage 75 and other
355 parameters as default.

356 The 28 unitigs from the HGAP assembly were BLASTed against the *P. chabaudi* AS v2
357 assembly¹⁴ to identify sequences relating to nuclear, mitochondrial and apicoplast
358 genomes. Four unitigs were found to be mouse contamination. Fourteen unitigs
359 represented the fourteen chromosomes from the v2 assembly including most of the
360 unplaced contigs from v2 and some additional new sequence. All fourteen had telomeric
361 repeats at both ends. The only major rearrangement between the assemblies was between
362 the ends of chromosomes 6 and 13. We mapped Quiver-corrected PacBio reads against
363 v2 and v3 assemblies and confirmed that the v3 assembly was correct. However, we
364 noticed base errors and in particular many indels in the v3 assembly due to the relatively
365 high error rate of PacBio sequencing. The v2 assembly had had the benefit of Sanger and
366 Illumina technologies such that at the base level it was more accurate than the PacBio
367 assembly. Therefore we generated 2x250bp paired-end pseudoreads from the v2
368 assembly with a fragment size of 600bp to a depth of 50x and used these to correct the v3
369 assembly using iCORN2⁴⁴. We transferred gene models from the v2 assembly to the
370 corrected v3 assembly using RATT⁴⁴. We then trained Augustus v2.5.5⁴⁵ using the v2
371 gene models with default parameters and predicted a new set of models for the corrected
372 v3 assembly. We kept only those, which did not overlap with the RATT-transferred gene

373 models. We then manually assessed the transferred models, annotated the new Augustus
374 models and renamed the locus tags. In general we added a zero to the end of the v2 locus
375 tags, but where a gene did not exist in v2 or had moved we added an odd number at the
376 end. We maintained numerical ordering of locus tags across each chromosome so that
377 they reflect relative chromosomal location.

378

379 **Sequence analysis of *pir* genes**

380 *Pir* gene sub clade assignments were initially made using Hidden Markov Models
381 (HMMs) built from the rodent malaria *pir* subclades published in Otto et al.¹⁴. There were
382 some discrepancies between the two sets, e.g. where the clade in the published tree did
383 not agree with the top HMM hit. In particular we identified three members of the S3
384 subclade, which was not previously thought to be present in *P. chabaudi*. We used
385 hmmsearch from HMMer i1.1rc3⁴⁶ with an E-value cutoff of 1e-10 and took the best hit
386 to a subclade. We thus identified 207 *pir* genes which we could assign to a rodent malaria
387 parasite (RMP) *pir* subclade, while a further five genes could not be classified into
388 existing subfamilies (PCHAS_0401400, PCHAS_0602000 and PCHAS_1247000).
389 HMM assignments of L2 *pirs* in *P. berghei* were consistent with the original tree-based
390 classification presented in Otto et al. (2014).

391 We used FIRE v1.1a⁴⁷ to identify putative promoter sequences upstream of genes in the
392 AAPLs and ChAPLs. Firstly we used RNA-seq data from the study to determine 5'
393 untranslated regions (UTRs) of *pir* genes and then manually identified untranscribed
394 regions upstream of each UTR, reaching until the proximal UTR of the next upstream
395 gene. Where there was insufficient RNA-seq data or other ambiguity, we did not identify

396 a region. This was successful for 27/32 ChAPL genes and 22/28 AAPL genes. We then
397 defined AAPL and ChAPL genes as two distinct expression clusters and ran FIRE with --
398 exptype=discrete and --nodups=1 options.

399 Sequence alignments were performed using Muscle⁴⁸ with default parameters and
400 visualized using Jalview⁴⁹.

401

402 **Regression analysis of *pir* genes against parasitological parameters**

403 DESeq2 was used to determine whether variation in gene expression between parasite
404 populations in different mice was correlated with parasitological parameters such as
405 parasitaemia and time of recrudescence⁵⁰. In particular we found that the expression level
406 during the acute phase of 152 genes was correlated with the time to recrudescence across
407 nine mice (p-value < 0.01; fold change >= 2). We discretized the time in days at
408 recrudescence as follows: mice 214 (32 days), 421 (31 days), 427 (30 days) were short,
409 2014 (34 days), 2114 (36 days), 452 (33 days) and 456 (34 days) were medium, 211 (43
410 days) and 212 (39 days) were long. We then used this single discrete factor in the
411 DESeq2 design formula. Those genes where expression level during the acute phase was
412 negatively correlated with time at recrudescence were, with a single exception, *pir* genes
413 from 3L, 6L and 6R subtelomeres (Table S6). These are the three ChAPLs and another
414 L1-rich locus (4R). Interestingly, the strongest effect was from an exported gene of
415 unknown function (PCHAS_1201300). The strongest positive effects were from *pir*
416 genes in AAPLs.

417

418 **Mixed infection**

419 Transgenic lines of *Plasmodium chabaudi* AS expressing mCherry or mNeonGreen under
420 the control of the constitutive promoter EF1 α were generated by transfection with the
421 plasmid *PcEFp230p_mCherry* and *PcEFp230p_mNeonGreen*, respectively. These
422 plasmids targeted the silent *230p* genomic locus, and were generated by replacement of
423 the ssu rRNA locus of *pPc-mCH_{CAM}*, described in⁵¹, with the *P. chabaudi* *p230p* locus
424 (*PCHAS_03_v3* 276,451-283,284). For *PcEFp230p_mNeonGreen*, the mCherry tag was
425 replaced with mNeonGreen⁵². Parasites were cycled ON and OFF acid water containing
426 pyrimethamine to select for stably transfected parasites, and cloned by serial dilution. The
427 insertion was verified by PCR amplification and Southern blot analysis and fluorochrome
428 expression by Flow cytometry analysis (Figure S9). mNeonGreen-expressing parasites
429 were then serially blood passaged (SBP) 10 times while mCherry-expressing parasites
430 were mosquito transmitted (MT) into donor mice. Both parasites were then mixed at
431 different ratios and 10⁵ infected erythrocytes were injected i.p. into ten recipient mice per
432 group. Competition between parasite lines as well as parasitaemia and pathology were
433 then monitored in recipient mice. To determine the ratio of mNeonGreen- and mCherry-
434 expressing parasites during infection, tail blood was collected and stained with Hoechst
435 33342 (New England Biolab). Samples were acquired on a BD LSRII flow cytometer and
436 data were analysed with FlowJo software (TreeStar).

437

438 **Clodronate liposomes treatment**

439 B6.RAG-1^{-/-} mice were treated with 200 μ L i.v. of either saline (Sigma; negative control)
440 or clodronate liposomes (clodronateliposomes.org). Flow cytometric analysis was
441 carried out to assess the depletion of phagocytic cells. Single-cell suspensions of

442 splenocytes were prepared, , and cells enumerated 24h after *in vivo* treatment. After
443 staining with Zombie Aqua (Biolegend) for live/dead discrimination, cells were stained
444 with monoclonal antibodies using appropriate combinations of fluorochromes (CD11c-
445 BV786, CD169-BV605, F4/80-BV421, Ly6G-PerCPCy5.5, MHCII-FITC, Ter119-PE-
446 Cy7, CD68-PE, Ly6c-APC-Cy7, CD11b-AF647, all from Biolegend). The samples were
447 acquired on a BD Fortessa/X20 (BD Biosciences) using Diva acquisition software (BD
448 Biosciences). FlowJo (Tree Star) software was used to analyze the data.

449 To determine the role of T-, B- and phagocytic cells in the selection of virulent parasites,
450 B6.RAG-1^{-/-} mice were treated with saline or clodronate liposomes as described above at
451 days -1 and 4 after i.p. infection with 10⁵ iRBCs containing a mix of Neon-Green-
452 expressing SBP parasites and mCherry-expressing MT parasites (see "Mixed infections"
453 section).

454

455 **Data availability**

456 RNA-seq datasets used in this study have been submitted to the European Nucleotide
457 Archive (ENA) under study accession ERP017479. These datasets are described further
458 in Supplementary Table 10. The Pacific Biosciences RSII genomic sequencing reads,
459 used to generate the *Plasmodium chabaudi chabaudi* AS v3 genome sequence, have been
460 submitted to the ENA under experiment accession ERX613966. The assembled genome
461 sequence and annotation can be accessed from GeneDB
462 (<http://www.genedb.org/Homepage/Pchabaudi>) and PlasmoDB
463 (<http://plasmodb.org/plasmo/>). The authors declare that no competing interests exist.

464

465 **References**

466

467

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614

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628

629 **Author contributions**

630 T.B., A.J.R., C.N., M.B. and J.L. (last author) designed the study. T.B. designed and
631 performed the mouse experiments with the help of I.T., G.K., S.M. and P.S.. A.J.R.
632 designed the sequencing experiments and performed the bioinformatic analyses. M.S.
633 coordinated sequencing experiments. U.B. manually annotated the *Plasmodium chabaudi*
634 AS genome sequence. D.C. and J.L. (7th author) created transgenic parasites. P.E.D. and
635 S.C performed thicket rat experiments, T.M, E.B. and O.B carried out the experiments in
636 the Brown Norway rats. T.B., A.J.R., C.N., M.B. and J.L. (last author) wrote the
637 manuscript.

638

639 **Competing financial Interests**

640 The authors declare no competing financial interests.

641

642

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644

645 Correspondence to Jean Langhorne, Thibaut Brugat and Adam Reid. Requests for
646 parasites, mice and reagents should go to Jean Langhorne. Requests for
647 sequencing/bioinformatic data should go to Adam Reid.

648

649 **Figure legends**

650 **Figure 1: Chronic infections modify the transcriptome of *P. chabaudi* independently**
651 **of the adaptive immune response.**

652 Parasitaemias over the course of infection in (A) 10 wild-type C57Bl/6 mice, (B) 10
653 B6.μMT^{-/-} mice and (C) 10 TCRα^{-/-} mice are shown on the left panels. Parasite mRNAs
654 were collected from 9 wild-type C57Bl/6 mice, 5 B6.μMT^{-/-} mice and 3 TCRα^{-/-} mice
655 selected randomly at the time points indicated by the red arrows. On the right panels, hot
656 pie diagrams show expression levels (black and white inner circles) and fold changes
657 (coloured outer circles) for genes expressed more highly (FDR ≤ 0.01, fold change ≥
658 2) in the chronic and acute phases. Genes are classified into groups according to several
659 categories including: subtelomeric genes (subtel.); genes associated with red blood cell
660 invasion (invasion); genes associated with gametocytogenesis (sexual) and *pir* genes
661 (highlighted in brown).

662

663 **Figure 2: Chronic infections are characterized by the expression of distinctive**
664 **clusters of *pir* genes in *P. chabaudi*.**

665 (A) Chronic Associated *Pir* Loci (ChAPL), and (B) Acute Associated *Pir* Loci (AAPL)
666 are shown in the context of the subtelomeric region from the telomeric repeats to the last
667 *pir* or *fam-a* gene. Those genes co-expressed across mice over the course of infection are
668 highlighted in orange. (C) Heatmap of *pir* gene expression at ChAPL and AAPL loci,
669 showing mean-normalised RPKM values for 10 wild type C57Bl/6 mice, four B6.μMT^{-/-}
670 mice and three TCRα^{-/-} mice infected by mosquito bites. Each column represents values
671 obtained from an individual mouse. Samples were collected from the same mice during

672 the acute and chronic (chro.) phases of infection. On the side, numbers indicate
673 chromosomes and letters indicate the location on that chromosome: L for left hand end, R
674 for right hand end. ChAPLs and AAPLs are highlighted. For each gene, red represents its
675 maximal expression, green its minimal expression. The absolute maximum and minimum
676 values for each gene are shown in a separate heatmap.

677

678 **Figure 3: Chronic infections select for virulent *P. chabaudi* parasites expressing**
679 **distinctive clusters of *pir* genes.**

680 Donor mice were infected by mosquito bites. (A) Parasites were subsequently passaged
681 into naïve recipient mice after one, three, four or six weeks (n=15 mice per group). (B)
682 Individual parasites were cloned during the acute and chronic phases of infection. After
683 their expansion, clonal populations of parasites were used to infect recipient mice (n= 5
684 mice per clone). In (A) and (B), we show maximum parasitaemia, temperature change
685 and weight change for recipient mice compared to mice directly infected by mosquito
686 bites (MT; blue dotted line) or with serially blood passaged parasites (SBP; red dotted
687 line). Each dot represents the mean \pm standard error of the mean (SEM) of 5 individual
688 mice. Each group has been compared to mice infected with 10^5 MT parasites (* P <0,05;
689 ** P <0,01; *** P <0,001, two-sided Mann Whitney Test). (C) Heatmap of *pir* gene
690 expression at ChAPLs and AAPLs in three acute and three chronic clones. The first three
691 columns represent cloned parasite lines derived from from the acute phase (f, a, b), the
692 next three columns represent cloned lines from the chronic phase (k, n, p) from 3
693 individual mice. On the side, chromosomes numbers are indicated as well as the location
694 on that chromosome: L for left hand, R for right hand. ChAPLs and AAPLs are

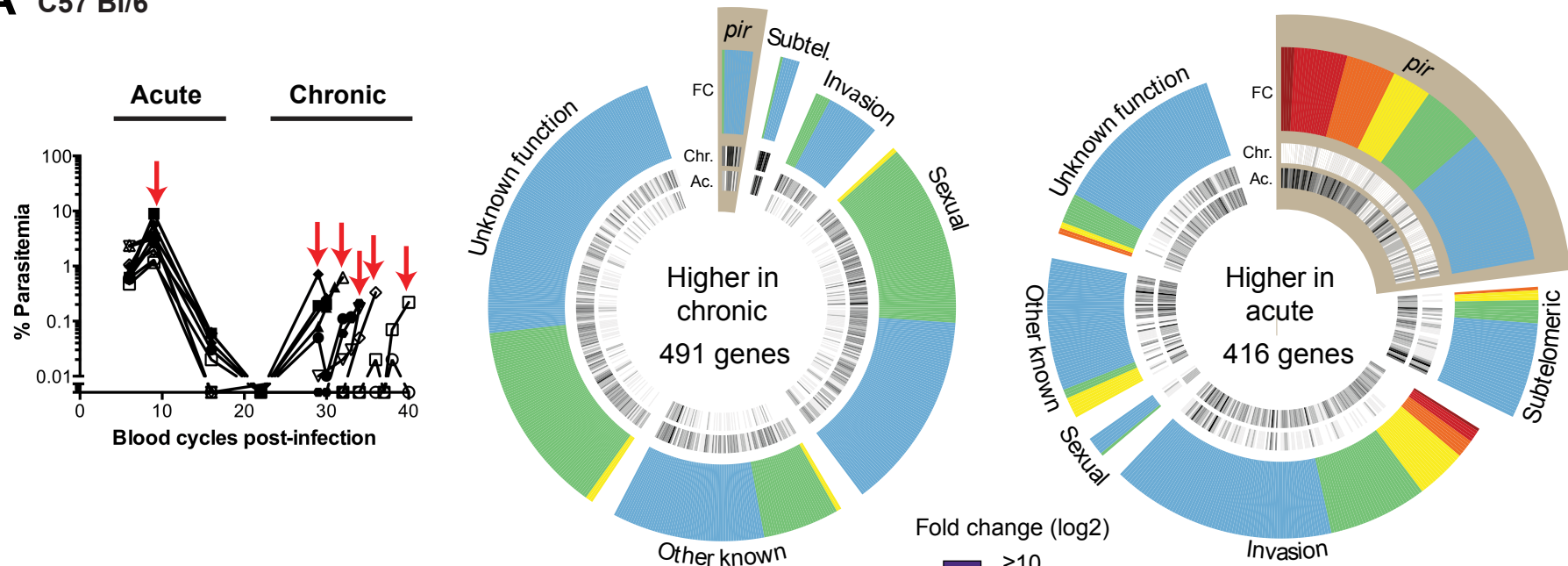
695 highlighted. For each gene, red represents its maximal expression, green its minimal
696 expression. The absolute maximum and minimum values for each gene are shown in a
697 separate heatmap.

698

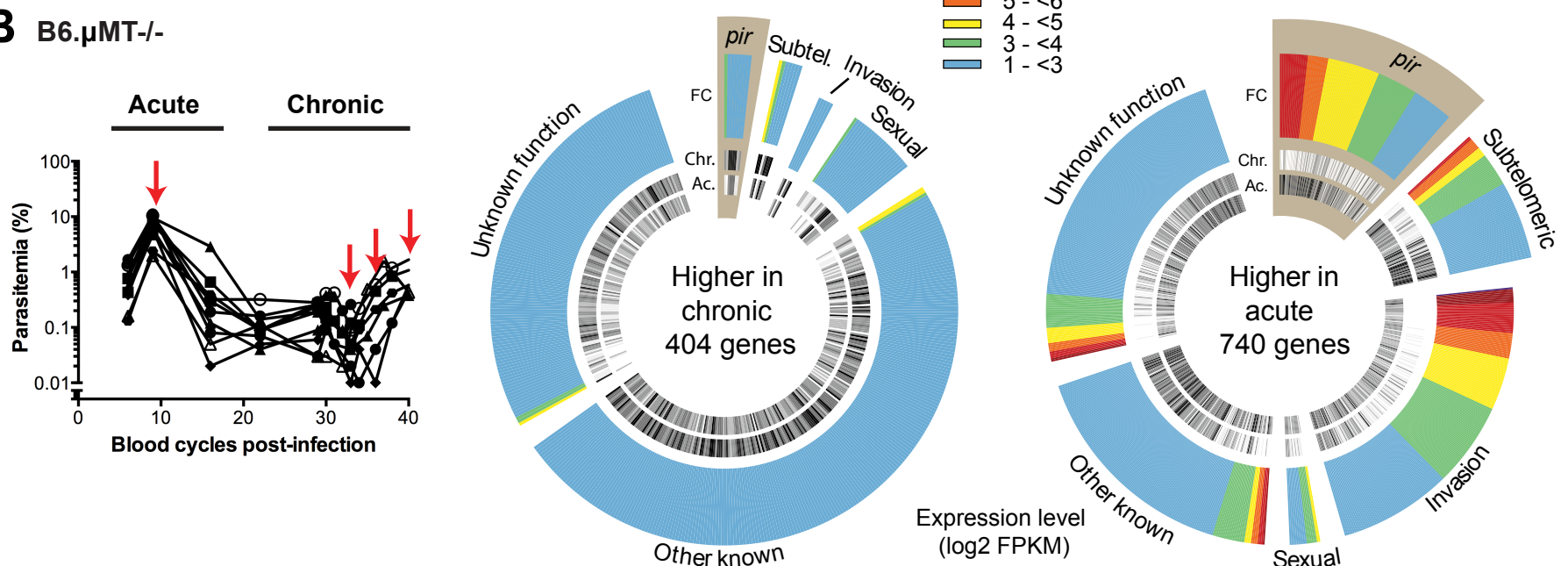
699 **Figure 4: Chronicity and virulence of infection are dictated by the initial**
700 **composition of *P. chabaudi* population.**

701 Parasites tagged with mCherry (Fluo 1) were Mosquito-Transmitted (MT), and
702 mNeonGreen (Fluo 2) tagged parasites were Serially Blood-Passaged (SBP). Different
703 proportions of the two were used to infect recipient mice by i.p. injection of 10^5 parasites
704 (n= 10 mice per group). As a control, a group of 10 recipient mice was infected by i.p.
705 injection of 10^4 SBP parasites. The mean percentage of parasites being SBP (as
706 determined by flow cytometry) is indicated (upper panel). We show maximum
707 parasitaemia, temperature change, weight change (middle panel) and time of
708 recrudescence (lower panel) for each group compared to mice infected with 100% MT
709 parasites (blue dotted line) or 100% SBP parasites (red dotted line). Each dot represents
710 the mean \pm standard error of the mean (SEM) for each group of 10 mice. Each group has
711 been compared to mice infected with 10^5 SBP parasites (* $P < 0,05$; ** $P < 0,01$;
712 *** $P < 0,001$, two-sided Mann Whitney Test).

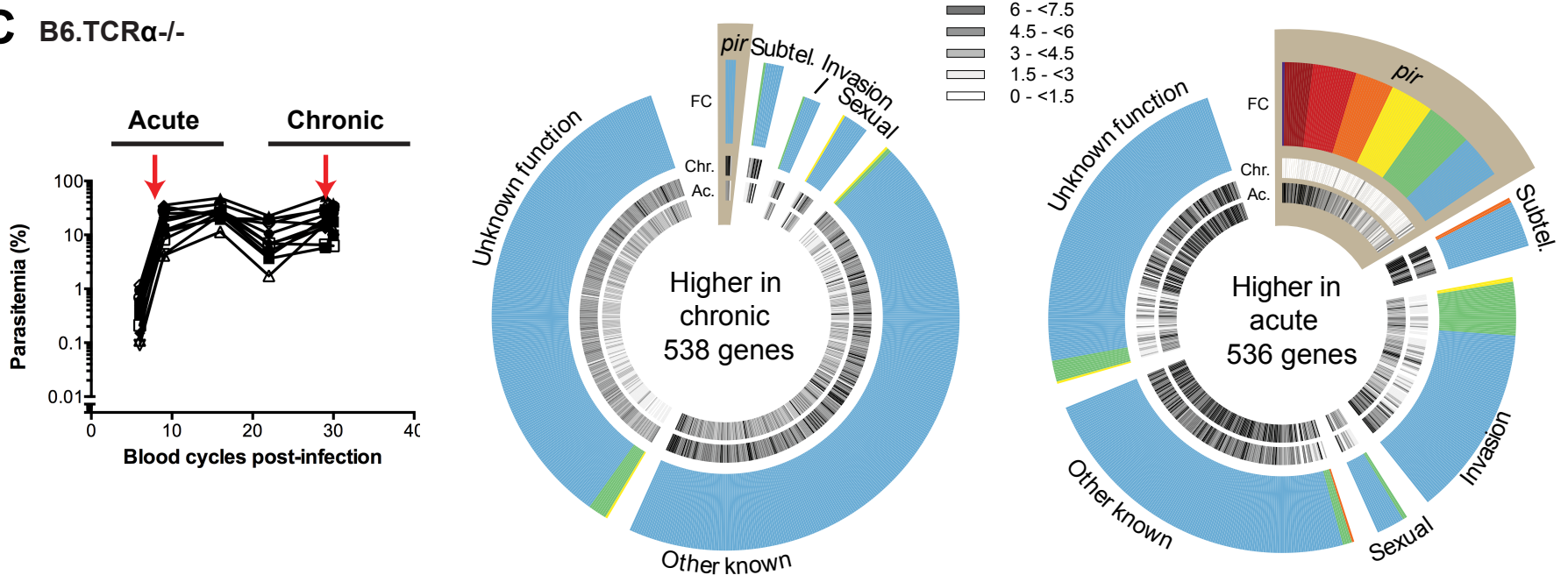
A C57 Bl/6

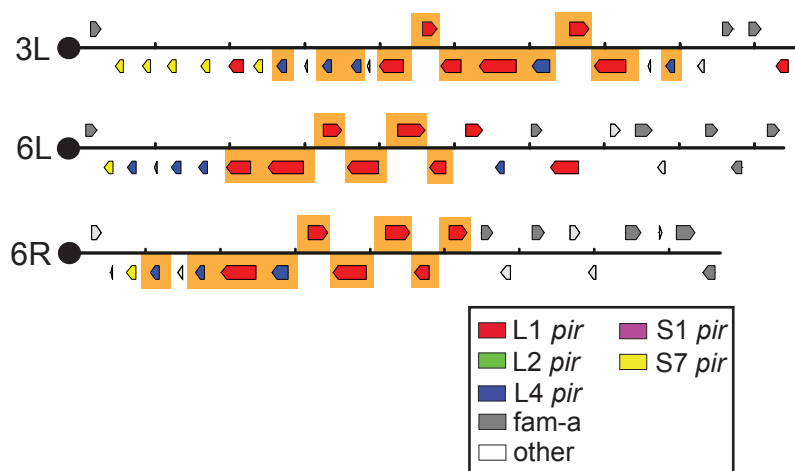
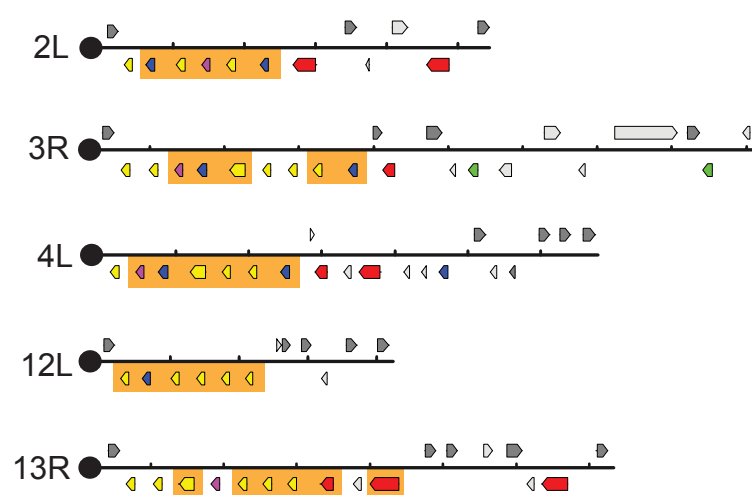
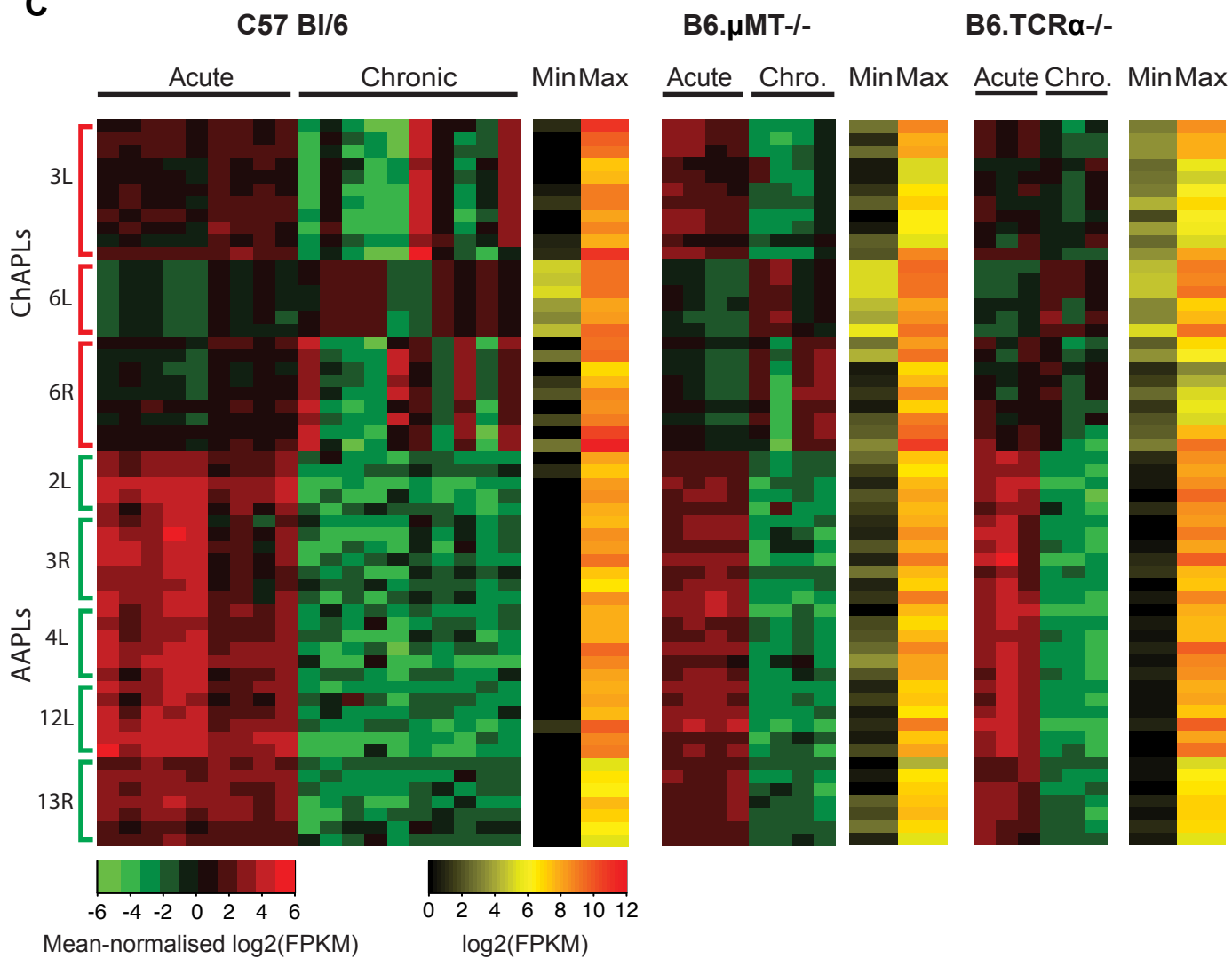


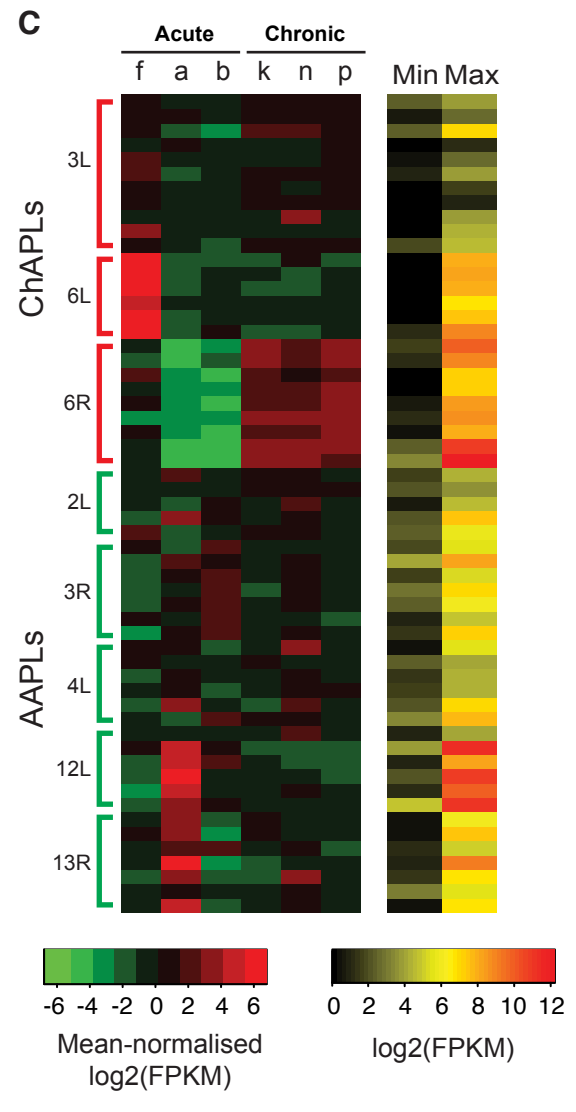
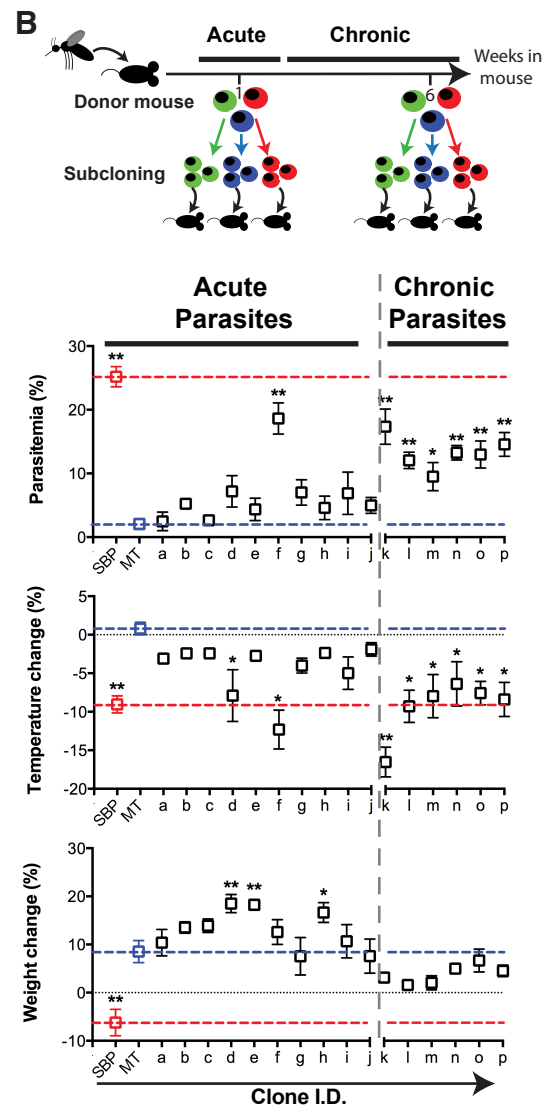
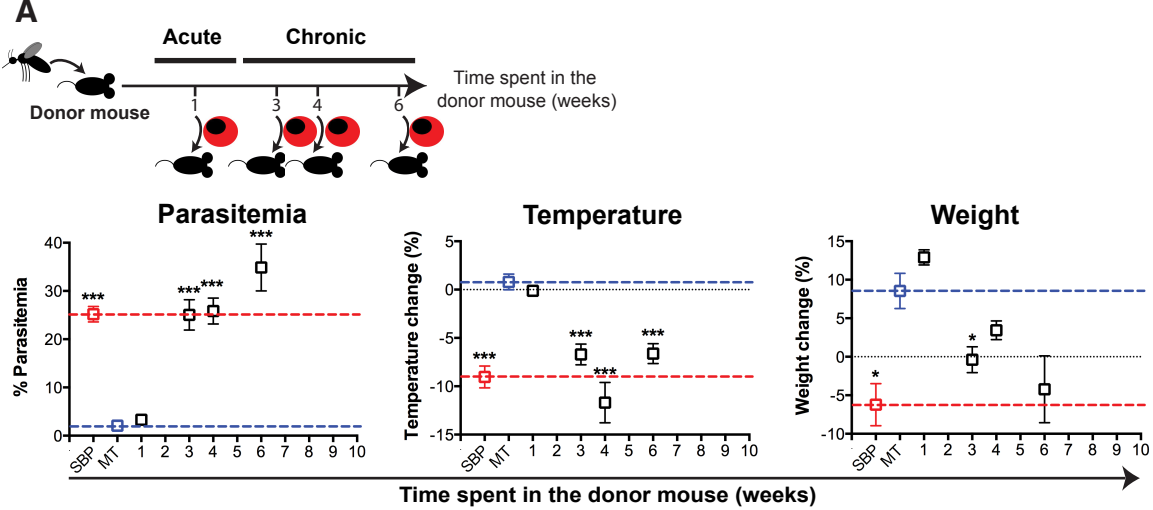
B B6.μMT^{-/-}

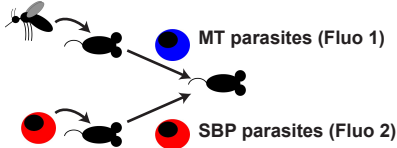


C B6.TCRα^{-/-}

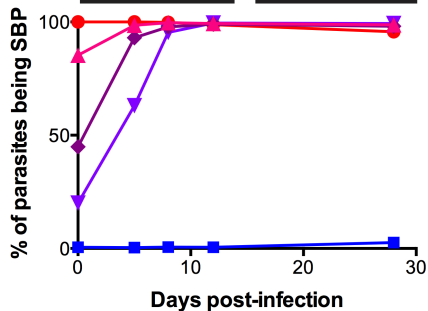


A ChAPLs**B AAPLs****C**





Acute **Chronic**



- 100% SBP (10^5 iRBC)
- 100% SBP (10^4 iRBC)
- ▲ 10% MT / 90% SBP
- ◆ 50% MT / 50% SBP
- ▼ 90% MT / 10% SBP
- 100% MT

