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Plasmodium falciparum genetic crosses in a humanized mouse model

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Abstract

Genetic crosses of phenotypically distinct strains of the human malaria parasite *Plasmodium falciparum* are a powerful tool for identifying genes controlling drug resistance and other key phenotypes. Previous studies relied on the isolation of recombinant parasites from splenectomized chimpanzees, a research avenue that is no longer available. Here, we demonstrate that human-liver chimeric mice support recovery of recombinant progeny for the identification of genetic determinants of parasite traits and adaptations.

Genetic crosses in the human malaria parasite *Plasmodium falciparum* have been outstandingly successful in locating the genetic determinants of important biomedical traits such as drug resistance and host specificity [1–3]. However, conducting genetic crosses with *P. falciparum* is technically difficult and expensive (Fig. 1a) and only three *P. falciparum* genetic crosses have been performed over a 28-year period. The biggest obstacle to routine generation of genetic crosses is the restriction of *P. falciparum* pre-erythrocytic stage development to human hepatocytes and chimpanzees [4] and recently the National Institutes of Health (NIH) has halted the use of chimpanzees for biomedical research. Thus, a new model for genetic crossing studies would be of immense value for malaria genetics research. Recently, we demonstrated that a human hepatocyte-liver chimeric mouse model (the FRG

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ACCESSION CODES

Whole genome sequences have been deposited to the NCBI Sequence Read Archive: SRP056319.

AUTHOR CONTRIUBTIONS

A.M.V., S.H.I.K. and M.T.F. conceived, initiated and supervised the project. A.M.V., S.H.I.K., T.J.C.A., R.S.P. and M.T.F. designed experiments and wrote the manuscript. A.M.V., N.C., M.F., L.A.C., R.S.P., I.A.C., S.N. and C.A.H. carried out the experiments. F.H.N. and T.J.C.A. supplied parasites. A.M.V., S.H.I.K., I.A.C., T.J.C.A., R.S.P. and M.T.F. analyzed results.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

huHep mouse) supports complete *P. falciparum* liver stage development, formation of exo-erythrocytic merozoites and transition to asexual blood stage replication when these mice are injected with human red blood cells (huRBCs) [5]. Consequently, we predicted that the FRG huHep mouse harboring huRBCs could be used as a novel and versatile vehicle for experimental *P. falciparum* crosses.

As a proof of concept, we initially staged a cross between the documented chloroquine resistant (CQR) strain, GB4 [6, 7] and a chloroquine sensitive (CQS) transgenic strain NF54HT-GFP–luc, which expresses a GFP-luciferase fusion [8] and is resistant to the human dihydrofolate reductase (DHFR) inhibitor, WR99210, due to transgene integration in the *pf47* locus on chromosome 13. Mature *in vitro* gametocyte cultures for both strains [9] were mixed for cross-fertilization and fed to *Anopheles stephensi* mosquitoes [10]. Sporozoites were isolated from mosquito salivary glands and approximately 4.0 million were injected intravenously into each of two FRG huHep mice. *In vivo* imaging demonstrated luciferase activity in the mouse liver indicative of parasite liver stage development (Fig. 1b) [8]. To allow for liver stage-to-blood stage transition, huRBCs were intravenously injected into the mice twice, at six and seven days following sporozoite injection, a regimen that maintains huRBC numbers in the mouse and enables initiation of *in vivo* blood stage development of transitioned parasites. Four hours after the second huRBC injection the mice were euthanized and exsanguinated to isolate the circulating *P. falciparum*-infected huRBCs. Giemsa-stained thin blood smears confirmed the liver stage-to-blood stage transition (parasitemia was <0.1% at this time point). We subsequently enriched infected blood with huRBCs and maintained in *in vitro* culture until parasitemia reached 1% at five days. As an initial screen for recombinant progeny, we treated blood stage cultures with WR99210 and CQ, reasoning that haploid parasites surviving this dual drug treatment would be recombinant having inherited independent drug resistant genes from each parent (mutant human *dhfr* from NF54HT-GFP–luc and the mutant *P. falciparum* chloroquine resistant transporter (*pfcrt*) on chromosome 7 from GB4). Individual progeny were cloned by limiting dilution and 10 progeny (lines derived from a single infected cell) were confirmed to each carry the integration in *pf47* (Fig. 1c, Supplementary Table 1) and the CQR mutant *pfcrt* (Fig. 1d, Supplementary Table 1). This result unambiguously demonstrates that the progeny were products of meiotic recombination.

We next performed a cross between NF54HT-GFP–luc and 7G8, a parasite line previously used in a cross to demonstrate the importance of the *pfrh5* gene in erythrocyte invasion [1]. Mature gametocyte cultures of each strain were mixed and fed to mosquitoes, and approximately 5.1 million sporozoites were injected into a FRG huHep mouse. We observed liver stage infection (Fig. 1b) and after liver stage-to-blood stage transition, *in vitro* blood stage parasites were cloned by limiting dilution three days after culture initiation. We genotyped 44 unselected progeny from this cross to evaluate Mendelian chromosomal assortment and recombination using an optimized a set of 22 polymorphic microsatellites (MS) [11] spanning all 14 parasite chromosomes (Fig. 2a, Supplementary Table 2). We saw a single allele at every locus genotyped confirming the clonality of all progeny lines. The 44 unselected progeny comprised 15 independent recombinants that were represented between one and eleven times (Fig. 2a) and each unique independent recombinant varied from all

other clones at minimally one marker. We found that six recombinant genotypes were repeated in multiple progeny (Fig. 2a). Of interest, we did not isolate any progeny with parental genotypes, indicating rare or non-existent selfing (male-female gamete pairing from the same parent). We next used rarefaction, a resampling approach widely used in ecology for estimating the numbers of species present in a community from a subsample, to estimate the total numbers of independent progeny present (Fig. 2a) [12]. These methods estimate 33 (95% CI: 19 – 96) independent recombinants for this cross, and that just over 150 clones would need to be sampled to attain this number.

Previous crosses in chimpanzees used parasites adapted for long-term laboratory rearing, but the utility of experimental genetic crosses will be most powerfully realized using fresh parasite isolates from clinical settings to study, for instance, emerging drug resistance [13]. To explore feasibility, we produced gametocyte cultures of a recent cloned field isolate (NHP*) [14] and crossed NHP* with NF54HT-GFP–luc. After injection of approximately 6.2 million sporozoites into a mouse, the infected liver showed a high luciferase signal (Fig. 1b). Indirect immunofluorescence assay (IFA) of liver sections taken after mouse exsanguination using an antibody to merozoite surface protein 1 (MSP1) confirmed a high infection rate in the mouse (Fig. 1b). After the liver stage-to-blood stage transition, a Giemsa stained smear of blood from the mouse showed a high level of huRBCs infected with *P. falciparum* ring stage parasites (Fig. 1b) and following only two days of *in vitro* blood stage culture, blood stage parasitemia reached 1%. MS analysis of the cloned progeny demonstrated the diversity of this cross: of 25 cloned progeny, we isolated 20 independent recombinants each carrying a unique allele combination (three clones carried only alleles from the NF54HT-GFP–luc parent and only two genotypes were represented twice among the cloned progeny) (Fig. 2a). Rarefaction curves estimated that 72 (95% CI: 36 – 198) independent progeny were generated (Fig. 2a), and this number of independent clones could be isolated from just over 100 cloned progeny lines.

To extend our genetic analysis further for the NF54HT-GFP–luc × NHP* cross, we sequenced the genomes of 14 independent recombinants and both parents. The parental sequences differed at 7536 high confidence single nucleotide polymorphisms (SNPs) (Supplementary Table 3), revealing 726 crossovers (Fig. 2b, Supplementary Fig. 1, Supplementary Table 3) in the progeny and a map length of 2210 cM (10.4 kb/cM) (Fig. 2c, Supplementary Table 3). The proportions of SNPs inherited from each parent centered on 50% in the progeny (Supplementary Fig. 2) consistent with Mendelian expectations, and the recombination parameters are comparable to those observed in the three previous crosses using chimpanzees [11, 15].

A large number of progeny carrying randomly assorting and segregating chromosomes, as well as ample recombination, are essential for high-resolution mapping of genes that confer phenotypes. From the three crosses we performed, 4, 15 and 20 independent progeny have been isolated (Fig. 2a, Supplementary Table 4). Rarefaction estimates based on the rate of discovery of new genotypes suggest that many more independent progeny can be recovered from the NF54HT-GFP–luc × NHP*cross. However, we isolated just four independent progeny from the initial NF54HT-GFP–luc×GB4 cross (Fig. 2a). This is likely in part due to the double drug selection regimen, which removes recombinants that do not carry both

resistance genes. In the other two crosses, where we did not employ drug selection, the estimated numbers of progeny that could be isolated are comparable (NF54HT-GFP–luc × 7G8) (Fig. 2a) or exceed (NF54HT-GFP–luc × NHP*) (Fig. 2a) those isolated from previous chimpanzee crosses [11, 15]. We speculate that the success in deriving large number of recombinants from the NF54HT-GFP–luc × NHP* cross (Fig. 2a, Supplementary Table 4), in part resulted from the use of a highly transmissible parasite that we recently isolated from a patient. Whole genome sequencing of 14 progeny from the NF54HT-GFP–luc × NHP* cross revealed recombination patterns characteristic of previous crosses conducted using chimpanzees, demonstrating the power of the FRG huHep mouse model to yield *P. falciparum* progeny for linkage mapping (Fig. 2b, Supplementary Fig. 1). Further optimization of the procedures described will improve the efficiency with which recombinant progeny are recovered.

The spread of artemisinin (ART) resistance in Southeast Asia [13] is an emergent crisis in malaria control. Population genetics and a five-year laboratory selection of ART resistant parasites led to the discovery of a marker for ART resistance, the Kelch locus [14, 16, 17]. The methodology presented here could have significantly accelerated the identification of this marker. Well-defined genetic crosses between ART resistant and susceptible strains now promise to improve our understanding of the genetic and functional underpinnings of ART resistance. Furthermore, staging *P. falciparum* genetic crosses using the FRG huHep mouse model provides a powerful framework for integrated analysis of multiple layers of genomic scale data (“Systems Genetics”) to better understand biomedically important parasite traits.

ONLINE METHODS

Study Approval

The study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH), USA. To this end, the Seattle Biomedical Research Institute has an Assurance from the Public Health Service (PHS) through the Office of Laboratory Animal Welfare (OLAW) for work approved by its Institutional Animal Care and Use Committee (IACUC). The PHS Assurance number is A3640-01. All of the work carried out in this study was specifically reviewed and approved by the Seattle Biomedical Research Institute IACUC.

FRG NOD huHep mice

Male and female FRG NOD huHep mice [18] with human chimeric livers were purchased from Yecuris Corporation. The mice are immunocompromised and should ideally be maintained in a barrier facility. In our hands, the mice are healthy throughout the course of experimentation. One mouse was used for each experimental genetic cross and the mice were not formally randomized or blinded before injection. The mice injected intravenously with *P. falciparum* sporozoites from the NF54HT-GFP–luc × GB4 cross and the NF54HT-GFP–luc × 7G8 cross were male and six months of age, and the mouse that received sporozoites from the NF54HT-GFP–luc × NHP* cross was female and seven months of age. The mice had human albumin levels between 4.3 and 7.3 mgs/mL. Mice used in the study

were supplemented with NTBC at 8 mg/L in their drinking water on arrival and maintained on this dose until euthanasia.

Plasmodium falciparum sporozoite production and liver stage infection

Anopheles stephensi mosquitoes (originating from the Walter Reed Army Institute of Research) were maintained at 27 °C and 75% humidity on a 12-hour light/dark cycle. Larval stages were reared following standard protocols as described in the MR4 manual with larval stages maintained on finely ground Tetramin fish food and adult mosquitoes maintained on 8% dextrose in 0.05% para-aminobenzoic acid (PABA) water.

In vitro P. falciparum blood stage cultures were maintained in RPMI-1640 (25 mM HEPES, 2 mM L-glutamine) supplemented with 50 μm hypoxanthine and 10% A+ human serum in an atmosphere of 5% CO_2, 5% O_2 and 90% N_2. Cells were subcultured into O+ erythrocytes. Gametocyte cultures were initiated at 5% hematocrit and 0.8 – 1% parasitemia (mixed stages) and maintained for up to 17 days with daily media changes.

Non-blood fed adult female mosquitoes three to seven days post-emergence were fed on mixed gametocyte cultures. Gametocyte cultures were quickly spun down and the pelleted infected erythrocytes diluted to a 40% hematocrit with fresh A+ human serum and O+ erythrocytes. Mosquitoes were allowed to feed through Parafilm for up to 20 minutes. Following blood feeding, mosquitoes were maintained for up to 19 days at 27 °C, 75% humidity and provided with 8% dextrose solution in PABA water. Infection prevalence was checked at days seven to ten by examining dissected midguts under light microscopy for the presence of oocysts with salivary gland dissections performed at days 14 – 19. In order to minimize the deleterious effects of salivary gland extract injection into the mice, average sporozoite numbers per mosquito were >30,000. Additionally, salivary gland dissection was performed to minimize the isolation of extraneous mosquito material.

Mice were injected intravenously into the tail vein with between approximately 4.0 and 6.2 million *P. falciparum* salivary gland sporozoites in 200 μL of RPMI media without supplements (Fig. 1). Six days after sporozoite injection, liver stage luciferase activity was visualized through imaging of whole bodies using the IVIS Lumina II animal imager (Caliper Life Sciences, USA). Mice were injected with 100 μL of RediJect D-Luciferin (Perkin Elmer) intraperitoneally prior to being anesthetized using an isofluorane-anesthesia system (XGI-8, Caliper Life Sciences, USA). Animals were kept anesthetized during the measurements, which were performed within 5 to 10 minutes after the injection of D-luciferin. Bioluminescence imaging was acquired with a 10 cm FOV, medium binning factor and an exposure time of 5 minutes. Quantitative analysis of bioluminescence was performed by measuring the luminescence signal intensity using the ROI settings of the Living Image® 3.0 software.

Liver stage-to-blood stage transition and *in vitro* culture of blood stages derived from FRG NOD huHep mouse infections

We have determined that the majority of liver stage parasites fully mature and release exoerythrocytic merozoites between 6 and 6 ½ days after sporozoite infection. To maximize

the number of transitioned ring stage parasites for downstream analysis mice are injected with huRBCs twice before exsanguination. Once, at six days after sporozoite injection and a second time at seven days after sporozoite injection. A second injection of huRBCs is performed to catch late transitioning liver stage parasites. Some huRBCs injected into the mice are cleared by the spleen, but the majority survive *in vivo* and thus liver stage parasites that mature and release exoerythrocytic merozoites after huRBC injection have the potential to invade huRBCs and begin to develop *in vivo* in the mouse. To this end, six days after sporozoite injection, mice were injected intravenously with 400 μL of packed O+ huRBCs. The intravenous injection was repeated on day seven. Four hours after the second huRBC injection, mice were sacrificed and blood was removed by cardiac puncture in order to recover *P. falciparum* infected huRBC. The blood was added to 10 mL complete media (RPMI-1640 with 25 mM HEPES, 2 mM L-glutamine, 50 μM hypoxanthine and 10% A+ human serum) and pelleted by centrifugation at 200*g*. The supernatant along with the buffy coat (containing white blood cells) were then removed and the red blood cells were washed three times with 10 mL complete media, with pelleting and centrifugation as detailed above. After the third wash, an equal volume of packed O+ huRBCs (approximately 400 μL) were added and the total RBC pellet was resuspended in complete media to 2% hematocrit. Cultures were split equally into six wells of a standard six well plate and maintained in an atmosphere of 5% CO_2, 5% O_2, and 90% N_2. Cultures were fed daily and 50 μL of freshly packed huRBCs were added every five days to each well. Once parasitemia reached 1%, serial dilutions of parasites were carried out to maintain healthy cultures. Additionally, cloning of parasites by limiting dilution was carried out in standard 96-well plates.

Immunofluorescence assay (IFA)

After mouse exsanguination at a time point when the majority of liver stage parasites have matured and released exoerythrocytic merozoites, the liver was perfused with phosphate buffered saline (PBS) through the hepatic portal vein, removed and separated into lobes and fixed in 4% electron microscopy grade formaldehyde in PBS, which was replaced by Tris buffered saline (TBS) + 0.05% (w/v) sodium azide after 24 hours. The fixed lobes were subsequently sliced into 50 μm sections for IFA, as detailed previously [19]. *P. falciparum* liver stages were detected with a mouse monoclonal merozoite surface protein 1 (MSP1) antibody (MRA-476) (1:1000 dilution). MRA-476 was obtained through the MR4 as part of the BEI Resources Repository, NIAID, NIH: *Mus musculus* (B cell); *Mus musculus* (myeloma) S1-3D6, MRA-476, deposited by CA Long. Human hepatocytes were detected with a rabbit polyclonal antibody (1:1000 dilution) to human fumaryl acetoacetate hydrolase (FAH) obtained from the Yecuris Corporation. Fluorescent Alexa Fluor 594 goat anti-mouse IgG and Alexa Fluor 488 goat anti-mouse IgG (life sciences) secondary antibodies were diluted 1:500. FAH has been deleted from the FRG mouse and thus mouse hepatocytes do not express FAH. MSP1 expression can be detected in both mature liver stage parasites and also at the localization of liver stage parasites that have previously released exoerythrocytic merozoites. This is due to the fact that residual MSP1 is seen at the site of merozoite release. Performing this IFA allows for an assessment of the success of the sporozoite infection and downstream liver stage development in the mouse. The DeltaVision Elite System 1 package was used for image capture following IFA. This encompasses an Olympus IX71 microscope to which is attached a Photometrics Cool SNAP HQ^2 camera. A 7 Color Combined

InsightSSI provided with the System provides ultra-fast solid-state illumination without filter changes. The image in Fig. 1B was captured using an Olympus UPlanFL N 10 × objective at room temperature. Use of the softWoRx 6.1.3 Software Suite allowed for image deconvolution and a single *xyz* image was pseudo-colored magenta (MSP1), turquoise (FAH) and blue (DNA) for the purposes of this publication.

PCR and RFLP analysis of clones from the NF54HT-GFP–luc × GB4 cross

Transitioned blood stage parasites were cultured with the addition of 50 nM CQ for five days and subsequently for a further seven days with 5 nM WR99210. The CQ and WR99210 resistant parasites were then cloned by limiting dilution and seeded at 0.5 parasites per well in 96 well plates. A total of 10 clones were initially recovered.

Genomic DNA from the NF54HT-GFP–luc and GB4 parents and recombinant progeny were isolated and subject to PCR using primers specific to the 5' and 3' *pf47* integration site for the GFP–luc expression cassette. Additionally, PCR primers that flanked the mutated allele within *pfcrt* were used for RFLP analysis following digestion with ApoI. Primers used for the study are listed in Supplementary Table 1.

Microsatellite analysis of recombinant progeny

Microsatellite (MS) markers were developed based on several criteria. All MS were first described in the high-resolution linkage map for *P. falciparum* [11]. Initially, 35 MS were selected for genome-wide representation and indication from the literature [6, 20] that they could detect sufficient polymorphism. These were evaluated to arrive at an optimized and informative set of 22 MS (Supplementary Table 2). The four parasites serving as parents (NF54HT-GFP–luc, GB4, 7G8 and NHP*) and the progeny resulting from the genetic crosses were grown under standard culture conditions [21]. Genomic DNA extracted from all the parasites using the phenol-chloroform method was PCR amplified using the Phusion Flash High-Fidelity PCR Master Mix (Thermo Scientific, Pittsburgh, PA) and fluorescent labeled primers (Sigma-Aldrich Corp., The Woodlands, TX) specific to the 22 MS markers distributed across the 14 chromosomes of *P. falciparum*. The PCR reaction mixtures were subjected to an initial denaturation at 98 °C for 10 s followed by 30 cycles of denaturation at 98 °C for 1 s, annealing at 48–58 °C for 5 s, extension at 65 °C for 15 s, and a final extension at 65 °C for 1 min. Primers for the MS markers used for genotyping are listed in Supplementary Table 2. The amplified products were analyzed on a CEQ 8000 Genetic Analysis System (Beckman Coulter Inc., Fullerton, CA).

Abundance of independent recombinants

We applied rarefaction, originally developed by ecologists to estimate the numbers of species present in a community, to estimate the number of recombinants generated by each cross using EstimateS v9.0.0 (http://viceroy.eeb.uconn.edu/estimates/). We used individual based curves and sampling without replacement to estimate the number of recombinants present. The results present were from Chao's formula 1, though identical results were obtained using the ACE estimator. In addition, we estimated the numbers of clones that would need to be sampled to obtain the estimated numbers of recombinants present.

Illumina sequencing and analysis of recombination parameters

Two μg of DNA was sheared using a Covaris S-series sonicator (Covaris; duty cycle 20%, time 180 s, intensity 5, cycle burst 200, power 37 W, temperature 7 °C, mode freq sweeping) from the two parents (NF54HT-GFP–luc×NHP*) and 14 recombinant progeny from the cross. Sheared DNA was end-repaired, A-tailed and multiplex-indexed adaptors ligated using NEBnext library preparation kits for Illumina (New England Biolabs). We replaced the DNA polymerase with Kapa HiFi (Kapa Biosystems) [22, 23] and used Agencourt AMPure XP beads (Beckman Coulter) for sample purification. The Kapa SYBR Fast ABI Prism qPCR kit (Kapa Biosystems) was used to quantify templates before multiplexing (13 samples/lane) and sequenced on an Illumina HiSeq 2500. Raw Sequence data was de-multiplexed and .fastq files generated using CASAVA 3.0 before further analysis.

101 bp paired-end reads from .fastq files were mapped against the *P. falciparum* genome reference strain 3D7 v9.2 (http://plasmodb.org/common/downloads/release-9.2/Pfalciparum3D7/fasta/data/) using BWA v0.6.1 [24]. The resulting BAM files were cleaned to remove reads which map off chromosomes and PCR duplicates removed using picard v1.56 (http://picard.sourceforge.net/). The Genome Analysis Toolkit v2.3-9 [25] was used to realign around indels and generate/re-calibrate base quality scores before final SNP calling was performed using the UnifiedGenotyper. Variant quality scores were then recalibrated and variants removed if they failed any of the following quality metrics (QUAL < 100.0, FS < 50, BaseQRankSum -2 > X > 2, MQRankSum -2 > X > 2, QD < 10).

A genetic map was constructed using the est.map function in R/qtl [26] in R v3.1.0 using the Haldane map function. As the parental lines were sequenced directly each SNP in the progeny was designated by its parent of origin. We calculated pair-wise allele sharing between progeny using the comparegeno function, and assessed if the observed data conformed to a normal distribution using the Shapiro-Wilk test for normality, implemented in the shapiro.test function in R.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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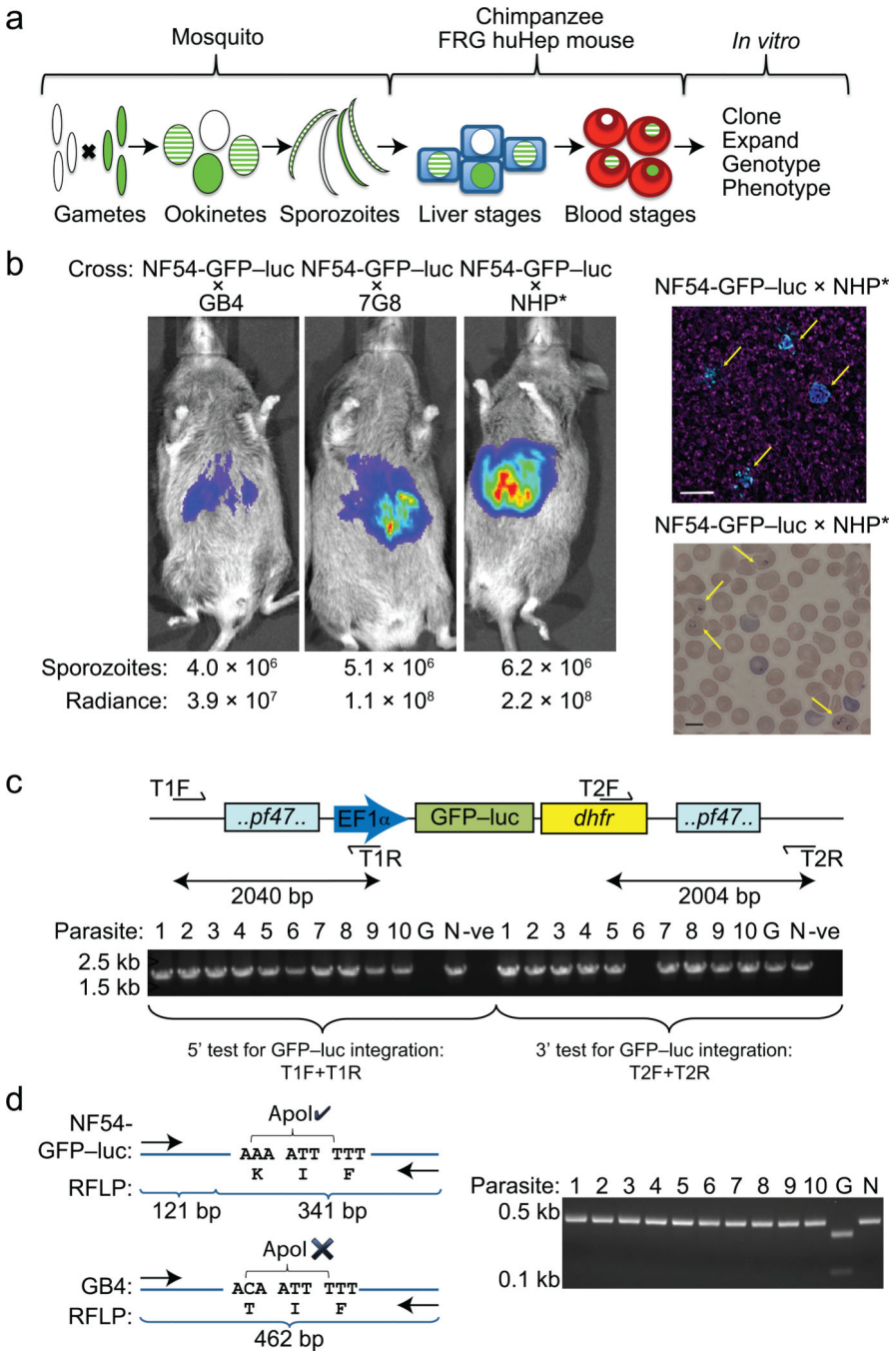

Figure 1. The FRG huHep mouse for *P. falciparum* genetic crosses

(**a**) Experimental crosses rely on parental gametocyte production (green and white), sexual recombination in the mosquito leading to ookinetes (green/white/striped) then sporozoites that are transmitted to FRG huHep mice, resulting in liver stage development and transition to blood stage replication. Cloning and expansion of recombinant progeny then allows for genotypic and phenotypic analysis. (**b**) Sporozoites from crosses between NF54HT-GFP–luc (NF54-GFP–luc) with strains GB4, 7G8 and NHP* were injected into FRG huHep mice and liver stage development was visualized on day six after inoculation by assaying luciferase

activity *in vivo* (left panel). Liver sections from the NF54HT-GFP–luc × NHP* cross mouse were subjected to IFA and a representative image shows the location of four matured liver stage parasites (yellow arrows) (merozoite surface protein 1 expression, magenta) in the highly humanized liver (fumaryl acetoacetate hydrolase expression, turquoise) (right, top panel). DNA (blue) was stained with 4', 6-diamidino-2-phenylindole. Scale bar: 100 μm. After liver stage to blood stage transition in the NF54HT-GFP–luc × NHP* cross, a representative Giemsa-stained smear showed substantial blood stage parasitemia (yellow arrows point to infected red blood cells) (right, bottom panel). (**c, d**). Progeny from the NF54HT-GFP–luc (N) × GB4 (G) cross were cloned after selection with WR99210 (NF54HT-GFP–luc is resistant) and chloroquine (GB4 is resistant). Ten cloned progeny contained both the human *dhfr* (hDHFR) cassette from the NF54HT-GFP–luc parent (**c**) and the chloroquine resistance (CQR) allele within *pfcrt* from the GB4 parent (**d**). Full-length gels are presented in Supplementary Fig. 3.

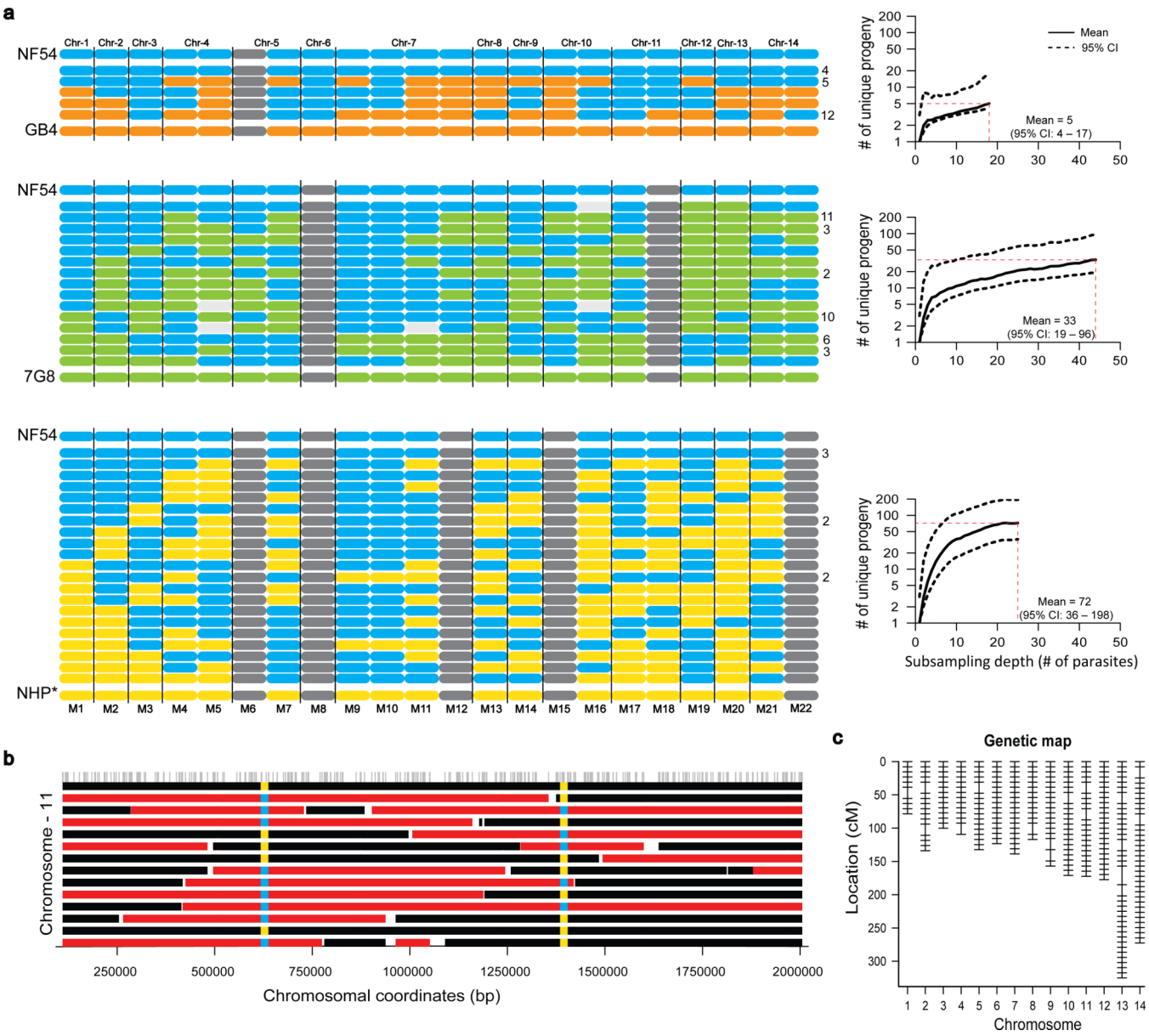

Figure 2. Analysis of recombinant progeny from *Plasmodium falciparum* crosses

(**a**) Genotyping results for clonal progeny obtained from three experimental *Plasmodium falciparum* genetic crosses between NF54HT-GFP–luc (blue) × GB4 (orange) (top panel), NF54HT-GFP–luc × 7G8 (green) (middle panel) and NF54HT-GFP–luc × NHP* (yellow) (bottom panel). 22 microsatellite (MS) markers were genotyped across the 14 chromosomes. Markers in dark grey indicate MS that were indistinguishable between the two parents. The number of cloned progeny carrying identical haplotypes is listed to the right of each haplotype; when a single haplotype was recovered, no number is listed. The rarefaction plots to the right estimate the total number of progeny present for each cross, with 95% confidence intervals marked by dashed lines. (**b**) Deep sequencing of parents and 14 of the progeny reveal detailed maps of recombination for the NF54HT-GFP–luc × NHP* cross (Supplementary Fig. 1), supporting the patterns of recombination shown by MS. Patterns of

inheritance for chromosome 11 are shown: see Supplementary Fig. 1 for all chromosomes. Haplotypes inherited from NHP* (black) or NF54HT-GFP–luc (red) are highlighted, with the genotypes of MS superimposed onto each chromosome. The grey tick marks along the top indicate the position of the segregating SNPs, while the scale (in base pairs) is shown at the base. (**c**) A high-density genetic map (Supplementary Table 3) from the NF54HT-GFP–luc × NHP* cross was constructed using deep sequence data.