Normocyte-binding protein required for human erythrocyte invasion by the zoonotic malaria parasite *Plasmodium knowlesi*

Robert W. Moon[a,b,1], Hazem Sharaf[c], Claire H. Hastings[a], Yung Shwen Ho[c], Mridul B. Nair[c], Zineb Rchiad[c], Ellen Knuepfer[a], Abhinay Ramaprasad[c], Franziska Mohring[b], Amirah Amir[d], Noor A. Yusuf[a,e], Joanna Hall[f], Neil Almond[f], Yee Ling Lau[d], Arnab Pain[c,g], Michael J. Blackman[a,h], and Anthony A. Holder[a,1]

[a]Mill Hill Laboratory, The Francis Crick Institute, London NW7 1AA, United Kingdom; [b]Department of Immunology and Infection, Faculty of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, London WC1E 7HT, United Kingdom; [c]Pathogen Genomics Laboratory, Biological and Environmental Sciences and Engineering Division, King Abdullah University of Science and Technology, Thuwal, Jeddah 23955-6900, Kingdom of Saudi Arabia; [d]Department of Parasitology, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia; [e]Parasitology Unit, Institute for Medical Research, Jalan Pahang, 50588 Kuala Lumpur, Malaysia; [f]Division of Retrovirology, National Institute for Biological Standards and Control, Health Protection Agency, Hertfordshire EN6 3QG, United Kingdom; [g]Research Center for Zoonosis Control, Global Institution for Collaborative Research and Education, Hokkaido University, Sapporo 001-0020, Japan; and [h]Department of Pathogen Molecular Biology, Faculty of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, London WC1E 7HT, United Kingdom

Edited by Janet Cox-Singh, University of St Andrews, St Andrews, United Kingdom, and accepted by Editorial Board Member Carolina Barillas-Mury May 11, 2016 (received for review November 13, 2015)

The dominant cause of malaria in Malaysia is now *Plasmodium knowlesi*, a zoonotic parasite of cynomolgus macaque monkeys found throughout South East Asia. Comparative genomic analysis of parasites adapted to in vitro growth in either cynomolgus or human RBCs identified a genomic deletion that includes the gene encoding normocyte-binding protein Xa (NBPXa) in parasites growing in cynomolgus RBCs but not in human RBCs. Experimental deletion of the *NBPXa* gene in parasites adapted to growth in human RBCs (which retain the ability to grow in cynomolgus RBCs) restricted them to cynomolgus RBCs, demonstrating that this gene is selectively required for parasite multiplication and growth in human RBCs. NBPXa-null parasites could bind to human RBCs, but invasion of these cells was severely impaired. Therefore, NBPXa is identified as a key mediator of *P. knowlesi* human infection and may be a target for vaccine development against this emerging pathogen.

invasion | zoonotic malaria | malaria parasite | normocyte-binding protein | *Plasmodium knowlesi*

A causative agent of severe zoonotic disease in South East Asia is *Plasmodium knowlesi*, a malaria parasite of the cynomolgus macaque, *Macaca fascicularis* (1, 2). In countries such as Malaysia where other malaria parasites are being controlled effectively, *P. knowlesi* infections are the dominant cause of human malaria (3, 4). In all malaria parasite species, disease is caused by cyclic parasite multiplication within RBCs, releasing invasive merozoites that invade new RBCs. Parasite adhesins transported onto the merozoite surface bind specific host cell receptors to facilitate RBC invasion (reviewed in ref. 5). The first adhesin–RBC interaction was identified in *P. knowlesi* and *Plasmodium vivax*, in which it was shown that parasites must bind to the Duffy antigen/chemokine receptor (DARC) to invade human RBCs (6). These adhesins are determinants of parasite virulence (7), host cell tropism (8), and potential vaccine candidates (9), and understanding the role of these adhesins may be critical for identifying the causes of increased risk of human infection and for the development of novel interventions.

Reticulocyte-binding proteins (RBPs) were first described in *P. vivax* (10) and are the prototypical examples of the reticulocyte binding-like/reticulocyte-binding homolog (RBL/RH) proteins also characterized in other malaria parasite species including *Plasmodium cynomolgi* (11) and *Plasmodium yoelii* (12). *Plasmodium falciparum* normocyte-binding proteins (NBPs) (13, 14) are members of this family that includes four conventional RBL/RH proteins and one unconventional short RBL/RH protein (RH5) in this species (reviewed in ref. 5). In *P. knowlesi* two proteins in the RBL family, NBPXa and NBPXb, have been identified (15), and recombinant fragments of these proteins bind RBCs (16). The RBL/RH adhesins and the Duffy-binding protein/erythrocyte-binding ligand (DBP/EBL) family of proteins play key roles during merozoite invasion of RBCs (reviewed in refs. 5 and 9). However, ascribing specific roles to these proteins has been hampered by functional redundancy, and only RH5 may be essential for *P. falciparum* invasion (17). In *P. knowlesi,* DBPα is the parasite adhesin binding DARC, and two paralogues, DBPβ and DBPγ, also have been identified (18), but thus far only DBPα has been shown to be required for RBC invasion (19).

P. knowlesi can be grown readily in both cynomolgus and rhesus (*Macaca mulatta*) macaque RBCs in vitro (20–22) but requires extended adaptation for growth in human RBCs in vitro, displaying insufficient invasion efficiency to support continuous culture in human RBCs. In previous work we overcame this

Significance

***Plasmodium knowlesi* is a parasite that naturally infects cynomolgus monkeys but is also a major cause of severe zoonotic malaria in humans in South East Asia. Comparing the genomes of parasites restricted to growth in culture with cynomolgus RBCs and those adapted to growth in human RBCs identified a gene specifically required for invasion of human RBCs, a process that is critical for parasite replication. This gene encodes normocyte-binding protein Xa, a protein previously shown to bind human RBCs and implicated in invasion. Disruption of this gene blocks invasion of human but not cynomolgus RBCs, thus confirming a key mediator of human infection and a potential target for inclusion in vaccines to prevent human infection.**

Author contributions: R.W.M., Y.L.L., A.P., M.J.B., and A.A.H. designed research; R.W.M., H.S., C.H.H., Y.S.H., M.B.N., Z.R., and A.A. performed research; E.K., F.M., N.A.Y., J.H., and N.A. contributed new reagents/analytic tools; R.W.M., H.S., Y.S.H., M.B.N., Z.R., A.R., A.P., M.J.B., and A.A.H. analyzed data; and R.W.M., A.P., M.J.B., and A.A.H. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission. J.C.-S. is a guest editor invited by the Editorial Board.

Data deposition: The sequences reported in this paper have been deposited in the European Nucleotide Archive (ENA) database [accession nos. CWHR02000001–CWHR02000037 (A1-H.1) and CWHQ02000001–CWHQ02000045 (A1-C.2)]. The raw FastQ files generated within this study are available under the following accession numbers: ERS763674 (A1-O), ERS763675 (A1-C), ERS763676 (A1-C.1), ERS763677 (A1-C.2), ERS763678 (A1-H), ERS763679 (A1-H.1), ERS763680 (A1-H.2), and ERS763681 (A1-H.3).

[1]To whom correspondence may be addressed. Email: Rob.Moon@lshtm.ac.uk or Tony.Holder@crick.ac.uk.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1522469113/-/DCSupplemental.

MICROBIOLOGY

impediment by extended adaptation in a mixture of human and cynomolgus RBCs, producing a parasite line that can be maintained in human RBCs alone (21). This previous analysis suggested that improved parasite invasion of human RBCs was essential for the maintenance of parasite growth in vitro.

Here, we undertook a comparative analysis of the genomes of *P. knowlesi* lines adapted to human or cynomolgus RBCs in culture, in parallel with analysis of the preadapted parasites. The comparison highlighted a crucial role for NBPXa in the invasion of human RBCs. Using the unique capacity to grow this parasite in two host cell types, we were able to disrupt the gene encoding NBPXa and show that it is required for invasion of human but not of cynomolgus macaque RBCs. Human-adapted *P. knowlesi* parasites provide a robust experimental platform to unravel the mechanisms of host cell invasion in both *P. knowlesi* and the related pathogen *P. vivax*.

Results

Culture Adaptation of *P. knowlesi* Is Associated with Distinct Large Genomic Deletions and Duplications. To identify the genetic changes occurring during adaptation to in vitro culture, we compared the parasite genomes at two critical points in the process: within 2 mo of introducing the parasite to culture (A1-O) and after full adaptation to growth in human RBCs (A1-H) or in cynomolgus RBCs (A1-C) for a similar period (Fig. 1*A*). Genome-sequence analysis confirmed that the starting A1-O parasite is closely related to the H strain (23, 24). Use of the Pacific Biosciences (PacBio) sequencing platform allowed the generation of high-quality genome assemblies (Table S1) for clones of the adapted parasite lines (A1-H.1 and A1-C.2) to use as references to identify SNPs and indels among both the uncloned lines and the other derived clones. Few nucleotide differences were identified (Fig. 1*A* and Dataset S1), with only 22 nonsynonymous variants in protein-coding genes throughout all the lines sequenced. Only three of these differences were found exclusively in the human-adapted parasite, and none was present in all clones, so they were unlikely to be responsible for the adaptation phenotype (Dataset S1). Similarly there were no nonsynonymous substitutions that were present in long-term culture adapted lines but absent from the A1-O parasite. In addition to these SNPs, we identified four larger deletions (3.6–66.3 kb) and one duplication (7.7 kb). In contrast to the SNPs, these appeared to be specific to either the human- or the cynomolgus-adapted lines, resulting in the deletion of 13 genes in A1-C–derived lines or the deletion of two genes and the duplication of four genes in A1-H–derived lines (Table S2). The largest deletion was of ∼66.3 kb at one end of chromosome 14 in the A1-C line (Fig. 1*B* and Fig. S1). In A1-O and A1-H lines, this region encompasses 12 genes (Table S2), including PKNH_1472300 which encodes NBPXa (15). This observation was of particular interest, because adaptation to human RBCs appeared to result from improved invasion efficiency rather than from improved intracellular growth (21). The genomic comparisons identified no substantial differences in other known invasion gene loci, including *DBPα*, *DBPβ*, *DBPγ*, and *NBPXb* genes. Similarly, although in A1-H derived lines we detected a duplication which contained four genes, including *GTP cyclohydrolase I*, we found no evidence of increased copy number for any of the *DBP* or *RBL* genes. However, using our data and a set of 48 *P. knowlesi* genomes from malaria infections in Malaysia (25), a high level of SNP diversity can be shown for the *NBPX* and *DBP* genes (Fig. S2 and Dataset S2).

Adaptation to Human RBCs Results in Increased Invasion Efficiency for both Human and Cynomolgus RBCs. The genetic differences between cynomolgus and human RBC-adapted parasite lines, including the loss of *NBPXa* in the A1-C line, suggested that the A1-C and A1-H lines had been subjected to distinct selection processes during culture adaptation. Therefore, the growth of these lines was compared with that of the A1-O line to try to correlate genetic and phenotypic differences (Fig. 2). To compare invasion efficiencies, schizonts purified from each line grown in cynomolgus RBCs were added to fresh human or cynomolgus RBCs, and then new intracellular parasites were quantified 24 h later. The invasion efficiency of the A1-O line in cynomolgus

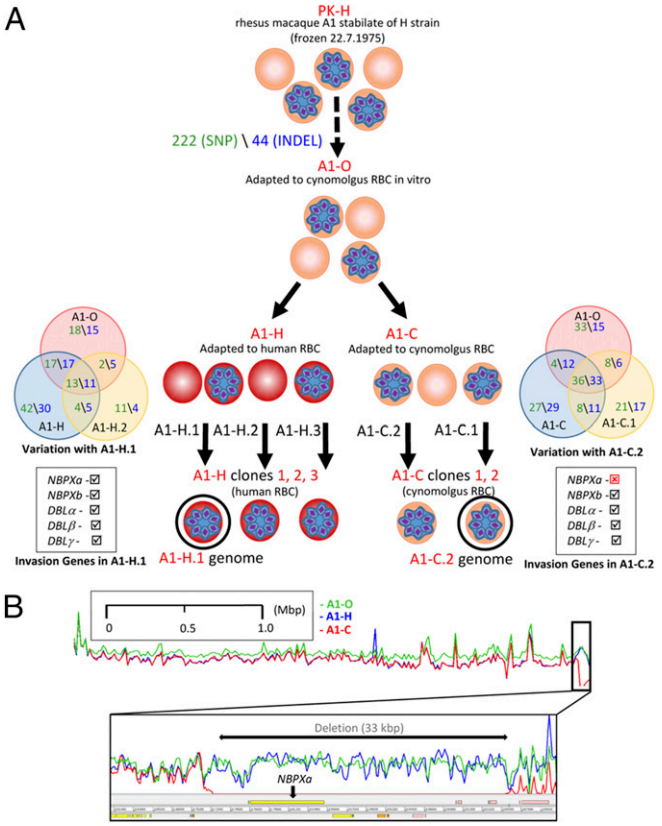

Fig. 1. Genomic comparisons of adapted parasite lines. (*A*) Genomic differences across *P. knowlesi* lines and derived clones used in this study. The starting material was the A1 stabilate derived from the H strain grown in a rhesus macaque. The schematic shows SNPs (green) and small indels (blue) identified between the H strain, the original A1-O line, and the human RBC-adapted A1-H and the cynomolgus RBC-adapted A1-C lines, and respective clones. Venn diagrams show the relationship among the A1-O, A1-H, and A1-C lines to the genome of a clone from each line. The presence of the known invasion genes in the clones (A1-H.1 to H.3, and A1-C.1 to C.2) is shown. (*B*) Mapping Illumina reads coverage of *P. knowlesi* A1-O (green), A1-C (red), and A1-H (blue) against chromosome 14 of *P. knowlesi* H viewed in Artemis. The enlarged area shows the lack of A1-C read coverage, corresponding to a 66.3-kbp deletion encompassing the invasion-related gene *NBPXa* (PKNH_1472300).

RBCs was around half that of both the A1-H.1 and A1-C.1 lines, as is consistent with selection for improved invasion following prolonged culture (Fig. 2*A*). Further examination of the cultures showed no evidence of defects in subsequent intracellular growth (Fig. 2 *B* and *C*). In human RBCs the invasion efficiency of the A1-O line was approximately half that of the A1-H.1 line but was four times that of the A1-C.1 line. Thus, the A1-H.1 line increased in invasion efficiency for both human and cynomolgus RBCs during more than a year in vitro, but the A1-C.1 line did so only in cynomolgus RBCs, with a reduction in invasion efficiency for human RBCs. Using the 24-h invasion data to calculate a mean host RBC selectivity ratio for the different lines (cynomolgus RBC invasion efficiency/human RBC invasion efficiency) revealed no significant differences between host cell selectivity of the A1-O and A1-H.1 lines (cynomolgus selectivity ± 1 SD = 1.53 ± 0.14 and 1.54 ± 0.49, respectively), clearly demonstrating that no specific increase in human RBC invasion efficiency had occurred in the A1-H.1 line. In contrast, the selectivity of the A1-C.1 line for cynomolgus RBC had increased significantly (cynomolgus selectivity = 16.1 ± 8.1) compared with both the A1-H.1 and A1-O lines ($P = 0.0044$, one-way ANOVA). The absence of *NBPXa* in the A1-C.1 line clearly did not reduce its invasion efficiency for cynomolgus cells. We therefore reasoned that its loss may have

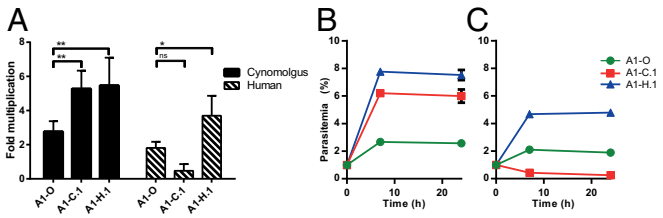

Fig. 2. Phenotypic characterization of the A1-O parasite line. (*A*) Growth of the A1-O line and the long-term culture-adapted A1-C.1 and A1-H.1 clones in cynomolgus or human RBCs after 24 h. The data shown are the mean of four independent experiments ± 1 SD. Statistical significance was determined using a two-factor ANOVA with the Sidak post hoc test to provide multiplicity-adjusted *P* values. Significant (*$P < 0.05$; **$P < 0.005$) and nonsignificant (ns) results are denoted. In cynomolgus RBCs both A1-C.1 ($P = 0.0040$) and A1-H.1 ($P = 0.0021$) lines grew significantly better than A1-O. In human RBCs only A1-H.1 ($P = 0.0285$) showed increased growth rate relative to A1-O; in contrast A1-C.1 demonstrated a nonsignificant but consistent reduction in growth relative to A1-O in human RBCs. (*B* and *C*) Parasitemia was determined after invasion (at 7 h) and after a cycle of intracellular growth (at 24 h) in cynomolgus (*B*) or human (*C*) RBCs, revealing that growth rates were determined by invasion efficiency and not by differences in subsequent intracellular growth. The data shown are the means of triplicate data points from a single experiment ± 1 SD.

caused the shift in host cell selectivity through reduced invasion efficiency for human RBCs. The ability of the A1-H.1 line to grow interchangeably in the two host RBCs offered a unique opportunity to test this hypothesis. No other clear relationship between genotype and adaptation to culture was established.

The *NBPXa* Gene Can Be Disrupted in A1-H.1 Parasites Grown in Cynomolgus but Not in Human RBCs. To analyze the role of *NBPXa*, A1-H.1 parasites were transfected with a gene-disruption construct (Fig. 3*A*) and were maintained under drug selection in either human or cynomolgus RBCs until resistant parasites emerged (8–15 d). Analysis of these parasites by PCR revealed successful disruption of *NBPXa* in parasites from independent transfections maintained in cynomolgus RBCs ($n = 5$) but not in parasites grown in human RBCs ($n = 3$), despite successful integration of control plasmid (Fig. 3*B*). Thus, the *NBPXa* gene appeared to be refractory to disruption in parasites grown in human but not in cynomolgus RBCs, indicating a host species-specific requirement for this gene. Two independent Δ*NBPXa* cloned lines were derived from A1-H.1 parasite transfections in cynomolgus RBCs and were used for subsequent phenotypic analysis.

***NBPXa* Is Unnecessary for Invasion of Cynomolgus RBCs but Is Crucial for Invasion of Human RBCs.** We examined the ability of the Δ*NBPXa* clones to invade and grow within either human or cynomolgus RBCs in the 24-h invasion assay. Disruption of *NBPXa* had no effect on invasion and growth in cynomolgus RBCs, but invasion of human RBCs was significantly impeded, matching the phenotype of the A1-C.1 line (Fig. 4*A* and Fig. S3). The defect in human RBC invasion caused by the loss of the *NBPXa* gene was severe and prevented multiplicative growth, but a low frequency of successful invasions was detected for both the A1-C.1 and *ΔNBPXa* lines. To rule out the possibility that this result was caused by contamination of purified schizonts with cynomolgus RBCs, the parasites were allowed to invade RBCs prelabeled with a fluorescent dye (26). The results showed that although contaminating cynomolgus RBCs contributed to a slight overestimation of invasion efficiency in human RBCs, only a small fraction of both the *ΔNBPXa* and A1C.1 parasites was able to infect human RBCs. Parasitemia measurements at 48 h showed that the *ΔNBPXa* human RBC invasion phenotype was expressed in subsequent cycles, and the parasite could not undergo multiplicative growth in human RBCs (Fig. 4 *B* and *C*). Extended culture of *ΔNBPXa* parasites in human RBCs resulted in a rapid reduction of parasitemia consistent with the severe defect. Although some parasites were detectable after 27 d of culture, these were revertants that had deleted the *NBPXa* disruptant construct (Fig. S3). These data demonstrate the severity of the *ΔNBPXa* phenotype, accounting for both the inability to disrupt the *NBPXa* gene and the severely reduced growth of the A1-C.1 line in human RBCs.

Further analysis showed that the *ΔNBPXa* block occurred within the first 7 h of the assay, as is consistent with a defect in invasion rather than in subsequent intracellular growth (Fig. 4 *B* and *C*); examination of Giemsa-stained blood smears taken at 7 h showed that although A1-H.1 parasites had successfully developed as ring stages in human RBCs, most of the *ΔNBPXa* parasites appeared to be extracellular merozoites that had attached to but had not invaded RBCs (Fig. 4 *D* and *E*). To see whether this observation was consistent with a phenotype expressed subsequent to RBC binding, we used an immunofluorescence-based merozoite surface protein 1 (MSP1) processing assay (27) to distinguish merozoites that had successfully invaded and formed rings ($MSP1_{19}$-positive only) from those that had attached but failed to invade, ($MSP1_{33}$ and $MSP1_{19}$ double positive) (Fig. 5*A*). Although 92% of A1-H.1 merozoites successfully formed rings in human RBCs, 76% of *ΔNBPXa* merozoites attached but failed to invade human RBCs (Fig. 5*B*). In contrast, about 90% of merozoites from both lines successfully formed rings in cynomolgus RBCs (Fig. 5*B*). These results show that *NBPXa* disruption results in a block in invasion following initial merozoite attachment.

To ensure that up- (or down-) regulation of another gene was not responsible for the observed phenotype, we performed RNA-sequencing (RNA-seq) analysis of schizont stages of A1-H.1 and *ΔNBPXa* parasites. This analysis did not identify any significantly differentially expressed genes.

Deletion of NBPXa Does Not Result in Restriction to Human Reticulocytes. Previous work by others had suggested that the ability to invade and grow in human reticulocytes as well as normocytes is a key parasite adaptation to culture in human RBCs (22). Our observation that both the *ΔNBPXa* and A1-C.1 lines invaded a small percentage of human RBCs raised the possibility that the loss of NBPXa function restricted the parasite to invasion of reticulocytes, which typically constitute 0.5–2% of total RBCs. To investigate this possibility, we

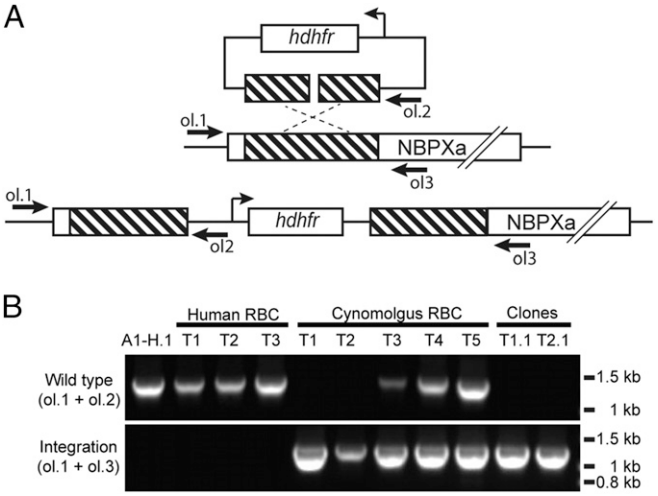

Fig. 3. Disruption of *NBPXa* can be achieved when parasites are grown in macaque RBCs but not in human RBCs. (*A*) Disruption of *NBPXa* by the introduction of linearized pNBPXa_1XKO into the genome via integration at the 5′ end of the *NBPXa* locus within a targeting region (hatched box). Primers for diagnostic analysis are indicated by thick black arrows. (*B*) PCR analysis of independently transfected parasites grown in human (T1 to T3) or cynomolgus (T1 to T5) RBCs and of two independent clones derived from the latter (T1.1 and T2.1) confirmed correct integration only for parasites grown in cynomolgus RBCs. (*Upper*) Amplification of a product from wild-type *NBPXa* (primers ol1 and ol2). (*Lower*) Amplification of a product from a species that can be produced only following integration (primers ol1 and ol3).

MICROBIOLOGY

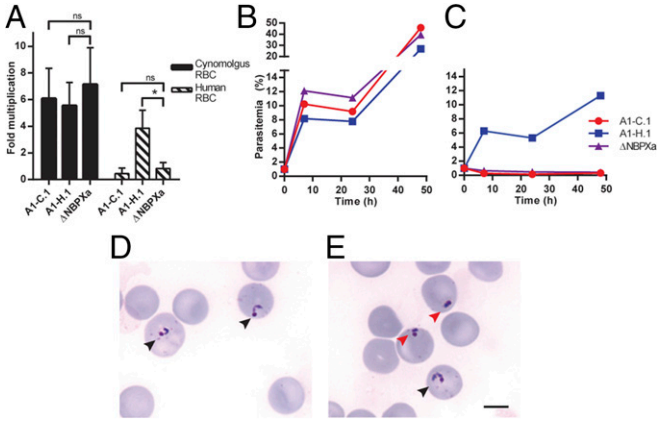

Fig. 4. Disruption of *NBPXa* prevents multiplicative growth in human RBCs through impaired invasion. (*A*) Growth of A1-C.1, A1-H.1, and Δ*NBPXa* in cynomolgus or human RBCs after 24 h. Data shown are the mean of four independent experiments ± 1 SD. Statistical significance was determined using a two-factor ANOVA with the Sidak post hoc test to provide multiplicity-adjusted *P* values. Significant (*) and nonsignificant (ns) results are shown. Compared with A1-H.1, Δ*NBPXa* showed no significant difference in growth in cynomolgus but a significant reduction in human RBCs (*$P = 0.0481$). The growth of Δ*NBPXa* and A1-C.1 did not differ significantly in human or cynomolgus RBCs. (*B* and *C*) Parasite growth and invasion over two cycles in either cynomolgus (*B*) or human (*C*) RBCs revealed a growth defect at or around invasion. Data shown are the means of triplicate data points from a single experiment ± 1 SD. (*D* and *E*) Light microscopic images of Giemsa-stained thin blood films from A1-H.1 (*D*) and Δ*NBPXa* (*E*) parasites in human RBCs at 7 h. Black arrowheads indicate newly formed rings; red arrowheads indicate attached but uninvaded merozoites. (Scale bar, 5 μm.)

used an adapted protocol (28, 29) to enrich reticulocytes to around 10% of human RBCs and used these cells for invasion assays. Reticulocyte enrichment resulted in a small increase in invasion efficiency over normal human RBCs for the A1-O, A1-H.1, A1-C.1, and *ΔNBPXa* lines, suggesting that all lines favor younger RBCs (Fig. S3). However, these increases were insufficient to explain the *ΔNBPXa* phenotype as the result of reticulocyte restriction. Furthermore, the few successfully invasive *ΔNBPXa* parasites were present in both reticulocytes and normocytes.

***NBPXa* Is Unnecessary for Invasion of Rhesus RBCs.** Disruption of *NBPXa* had no effect on invasion into rhesus RBCs (Fig. S3), although the invasion efficiency for all lines tested (A1-H.1, A1-C.1, and *ΔNBPXa*) was significantly higher in cynomolgus RBCs than in rhesus RBCs, suggesting that the parasite has slightly different adhesin specificities even for these closely related hosts. However this difference also may be explained by phenotypic culture adaptation to cynomolgus RBCs.

Discussion

In this work we set out to identify parasite features that facilitate growth in human RBCs in vitro, exploiting *P. knowlesi* lines that we previously adapted to growth in either human or cynomolgus RBCs. Comparative genome analysis of the culture-adapted lines revealed relatively few nonsynonymous SNPs, and none of these associated universally with either adaptation to human RBCs or long-term culture. In contrast, we detected several large genome deletions and a duplication that were present in either human- or cynomolgus-adapted lines but not in both and that were present in the uncloned lines as well as in clones derived from them, suggesting that selection had led to fixation within the population. Further analysis of these deletions either by individual gene deletion, as we have demonstrated for *NBPXa*, or by gene complementation may be informative. The duplication in A1-H–derived lines contains the gene coding for GTP cyclohydrolase, a rate-limiting enzyme in folate metabolism, and duplications of this gene are associated with drug resistance in South East Asian strains of *P. falciparum* (30). Increased activity in this pathway and metabolic adaptation may provide a general selective advantage for growth in culture.

Correct assembly and read mapping for several key gene families, in particular the DBPs, was only possible with the use of PacBio sequencing, which produces longer read lengths. Using these data as a scaffold to map the higher-coverage but shorter Illumina reads facilitated high-quality de novo assemblies of both A1H.1 and A1-C.2 cloned lines, which complement the existing H-strain data and will benefit further work with the culture-adapted *P. knowlesi* lines.

Our previous comparison of cynomolgus- and human-adapted *P. knowlesi* parasite lines had suggested that an increase in human RBC invasion efficiency enabled the parasite to adapt to long-term culture in human RBCs (21). However, the additional comparison with the invasion characteristics of the A1-O line, which had been in culture for less than 2 mo, demonstrated that the host cell specificity of the A1-H lines had remained unchanged. Instead the overall invasion efficiency for both human and cynomolgus RBCs had improved, with invasion of human RBCs being above the minimum threshold required to support in vitro multiplication and growth. In contrast, the invasion efficiency of the A1-C line (and its derived clones) increased only for cynomolgus RBCs. This increased selectivity is likely the result of the loss of a section of one end of chromosome 14 containing the gene for NBPXa, which we have demonstrated to be crucial for human RBC invasion. Given that the cynomolgus RBC invasion phenotypes of the A1-C.1 and A1-H.1 lines are indistinguishable, it is quite possible that if A1-C parasites had not lost *NBPXa*, their invasion efficiency for human RBCs in culture would have risen to levels equivalent to that seen for A1-H.1 without prior exposure to human RBCs. In principle this idea could be investigated by genetic complementation of the lost *NBPXa*

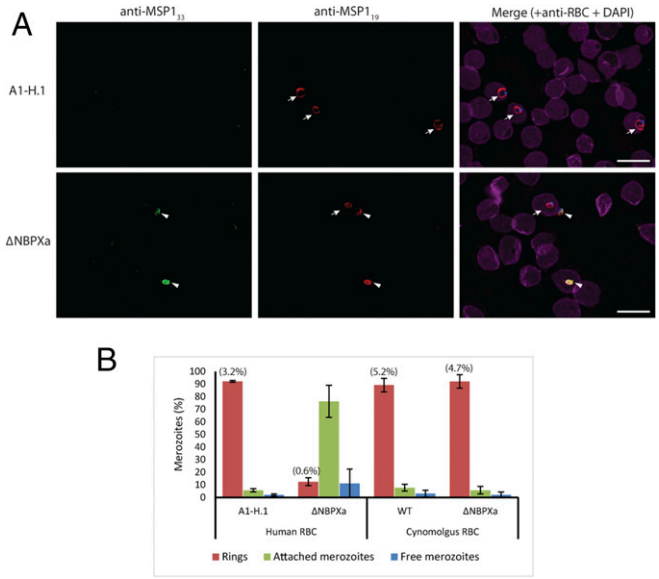

Fig. 5. Δ*NBPXa* parasites attach to human RBCs but fail to invade. (*A*) A1-H.1 and ΔNBPXa schizonts were purified, and released merozoites were allowed to invade human or cynomolgus RBCs for 3 h. Panels show blood smears stained with anti-MSP1$_{33}$ (green), anti-MSP1$_{19}$ (red), and a merge of both MSP1 antibodies with anti-human RBC (magenta) and DAPI (blue). MSP1 is processed during invasion, with MSP1$_{33}$ being shed and MSP1$_{19}$ retained, distinguishing external attached merozoites (both MSP1$_{33}$- and MSP1$_{19}$-positive) from those that had successfully invaded to form rings (only MSP1$_{19}$-positive). Although A1-H.1 merozoites (*Upper Row*) were able to invade human RBC and form rings (white arrows), most ΔNBPXa merozoites (*Lower Row*) attached but did not invade human RBC (white arrowheads). (Scale bars, 10 μm.) (*B*) The parasite stages present in the immunofluorescence assays were quantified. The graph shows the number of free merozoites, RBC-attached merozoites, and rings stages expressed as a percentage of total merozoite-derived forms counted. Data shown are the means of triplicate cultures from a single experiment ± 1 SD. The numbers in parentheses indicate the average ring-stage parasitemia for each of the parasite/blood combinations.

gene within the A1-C.1 line, but so far such efforts have been hampered by its large size (8.5 kb). The reasons for the different behavior of the H-strain parasite [which was isolated from a patient (23)] in vivo and in vitro are unclear, but our results suggest that for successful parasite multiplication and survival in vitro, prolonged physiological adaptation to culture is more important than adaptation to a specific host RBC. Our data identified clear differences in invasion efficiencies into RBCs from rhesus and cynomolgus macaques; this finding is surprising, given that these two primate species are closely related (31) and that experimental infection of rhesus monkeys (not a natural host of *P. knowlesi*) results in hyperparasitemia (23), in contrast to the chronic low-level parasitemia characteristic of *P. knowlesi* infection in cynomolgus macaques. Recombinant NBPXb expressed on cells binds cynomolgus RBCs four times more effectively than rhesus RBC (16), offering one possible mechanism for this difference in invasion efficiency. Independently established culture-adapted parasite strains developed using rhesus RBCs (20, 22) may have undergone adaptive changes quite different from those observed for cynomolgus RBCs. It also will be of interest to determine the contribution of metabolic adaptation to growth in different RBCs in culture.

P. knowlesi is phylogenetically closely related to *P. vivax* (32), a malaria parasite that causes the majority of malaria cases outside of Africa and that currently cannot be cultured in vitro. Although both these parasites use an orthologous DBP to bind to the DARC receptor on host RBCs (6, 18, 33, 34), there are greater differences in the RBL family. *P. vivax* can invade only reticulocytes, a phenotype attributed to the presence of RBP1 and RBP2, which bind only to reticulocytes (10) and are not conserved in *P. knowlesi*. Orthologs of the *P. knowlesi* RBLs are present in *P. vivax* (35, 36), but, given that our data indicate the *P. knowlesi* NBPXa protein is functional in invasion of both normocytes and reticulocytes, it is tempting to speculate that the absence of this protein in *P. vivax* could account for the parasite's restriction to reticulocytes. Intriguingly another closely related simian malaria parasite, *P. cynomolgi*, contains orthologs for RBL proteins specific for both *P. vivax* and *P. knowlesi* (11, 37). Transferring orthologs from *P. vivax* or *P. cynomolgi* into *P. knowlesi* may offer opportunities to study both the requirements for and the evolution of *P. vivax* invasion.

The unique advantages of the *P. knowlesi* culture system have enabled us to demonstrate that NBPXa plays a crucial role in the invasion of human RBCs. Its role appears to occur after initial merozoite attachment, perhaps during establishment of the moving junction between RBC and parasite plasma membranes, which is required for invasion. In other *Plasmodium* species both the RBL and DBP protein families are highly redundant, and ascribing precise roles has been difficult, although in *P. falciparum* they have been implicated in triggering signaling and release of secretory organelles (38). The ability to grow *P. knowlesi* interchangeably in different host cells with distinct adhesin requirements enables the generation of lines with gene knockouts for important parasite adhesins such as NBPXa, providing a powerful and unique opportunity to investigate their precise role in red cell invasion.

The adhesin repertoire is smaller in *P. knowlesi* than in many malaria parasites (9, 15, 18), and the loss of either *DBPa* (6, 19) or *NBPXa* creates a critical block in the invasion of human RBCs, perhaps because the paralogues cannot bind human RBCs. For example, although PkNBPXa and DBP*a* proteins bind to both human and macaque RBCs, DBPβ, DBPγ, and NBPXb bind only to macaque RBCs (16, 39). This apparent lack of adhesin redundancy for human RBC invasion suggests that *P. knowlesi* is vulnerable to perturbation of DBPα/NBPXa function. Although this lack of redundancy may encourage the use of these antigens in vaccine development, their diversity in the parasites responsible for human infection (25, 40) provides an important caveat. Vaccine development may be an important strategy for control of *P. knowlesi* malaria, given the presence of a primate reservoir in Southeast Asia.

NBPXa polymorphisms are important determinants of high parasitemia and disease severity in *P. knowlesi* infection (7) and may impact the potential for human-to-human transmission. Because we have shown a critical role for this protein in human RBC invasion, it is possible that the increased virulence and multiplication rates of parasites containing particular alleles result from an improved ability to bind and invade human RBCs. However, even the human-adapted *P. knowlesi* line invades cynomolgus RBCs more efficiently than human RBCs, suggesting that invasion efficiency may remain a bottleneck in human infections. Interestingly, variation in NBPXb, which had been found not to bind to human RBCs, also has been linked to disease severity in humans (7, 16). The role of NBPXb and variants of both *P. knowl*esi NBPs now can be investigated in vitro. Human-adapted *P. knowlesi* parasites provide a robust experimental platform to unravel the mechanisms of host cell invasion and to aid in the development of strategies to control malaria caused by *P. knowlesi* and the closely related pathogen *P. vivax*.

Methods

Macaque and Human RBCs. Cynomolgus and rhesus blood was collected by venous puncture. Animal work was reviewed and approved by the local National Institute for Biological Standards and Control Animal Welfare and Ethical Review Body (the Institutional Review Board) and by the United Kingdom Home Office as governed by United Kingdom law under the Animals (Scientific Procedures) Act 1986. Animals were handled in strict accordance with the "Code of Practice Part 1 for the housing and care of animals (21/03/05)" available at https://www.gov.uk/research-and-testing-using-animals. The work also met the National Centre for the Replacement Refinement and Reduction of Animals in Research (NC3Rs) guidelines on primate accommodation, care, and use (https://www.nc3rs.org.uk/non-human-primate-accommodation-care-and-use), which exceed the legal minimum standards required by the United Kingdom Animals (Scientific Procedures) Act 1986, associated Codes of Practice, and the US Institute for Laboratory Animal Research Guide. Human blood (Groups A^+ and O^+; $Duffy^+$) was obtained from the United Kingdom National Blood Transfusion Service.

Parasite Lines and Maintenance. *P. knowlesi* parasite cultures were derived from a frozen in vivo stabilate (A1, dated July 22, 1975) supplied by Louis Miller, National Institutes of Health, Bethesda, MD, and were obtained following passage in vivo in rhesus macaque of the *P. knowlesi* H strain derived from a clinical infection (23). A1-O is a nonclonal cultured line from the A1 stabilate propagated in cynomolgus RBCs in vitro for less than 2 mo. A1-H is a nonclonal line derived from A1-O following maintenance in 80% human and 20% cynomolgus RBCs for 10 mo and for up to 7 mo in human RBCs alone, with clones A1-H.1, A1-H.2, and A1-H.3 derived at this time. *P. knowlesi* A1-C is a nonclonal line derived from A1-O after maintenance in 100% cynomolgus RBCs for 15 mo, with clones A1-C.1 and A1-C.2 derived at this time. Parasites were grown and synchronized as described previously (21).

***P. knowlesi* Genome Sequencing, Analysis, and de Novo Assembly.** DNA for the A1-H.1 and A1-C.2 lines was used for preparation of libraries: HiSeq 2000 (Illumina, Inc.) compatible genomic shotgun libraries (500-bp fragment size); PCR-free libraries sequenced on MiSeq (Illumina); and, for genome assemblies, 20-kbp insert libraries sequenced on a PacBio RS-II instrument (Pacific Biosciences Inc.). Genome assembly was performed using PacBio's FALCON tools and Genome Consensus suite. Annotation was transferred from the *P. knowlesi* H strain reference genome (24), and gene models were checked in Artemis BAM view and corrected using strand-specific RNA-seq datasets.

Adhesion, Invasion, and Growth Assays. Purified schizonts were used to set up triplicate cultures with cynomolgus, rhesus, and human or human reticulocyte-enriched RBCs, at a 2% hematocrit and 1% parasitemia. All parasites (including A1-H.1) were grown in cynomolgus RBCs for at least 1 mo before growth assays to control for any effect of prior host cell type on subsequent invasion and growth. Parasitemia was measured with a flow cytometry (FACS)-based assay (21, 26) at before and after incubation at 37 °C in a gassed chamber for 7, 24, or 48 h. Samples were fixed, washed, permeabilized with Triton X-100, and then washed again before RNase treatment, staining with SYBR Green I (Life Technologies), and FACS analysis. To control for invasion of RBCs carried over during schizont purification, target cells were prelabeled with a far red fluorescence dye (26) using 7-hydroxy-9H-(1,3-dichloro-9,9-dimethylacridin-2-one) succinimidyl ester (DDAO-SE) (Life Technologies). Following washing the samples were analyzed on a CyAN ADP Analyzer (Beckman Coulter). Data were acquired and analyzed using CellQuest (BD) or HyperCyt (Beckman Coulter) software.

To investigate invasion/binding phenotype and reticulocyte preference, samples taken at 7 h were incubated with reticulocyte stain solution (Sigma).

Then the cells were pelleted, and Giemsa-stained thin blood films were prepared. To attempt to readapt ΔNBPXa parasites to growth in human RBC, a culture at 1% parasitemia was set up and maintained for 1 mo. After parasites were no longer detectable in blood smears (4 d), the culture was maintained by replacement with 30% fresh human RBCs and medium every 2–3 d. The (revertant) parasites that were detectable after 28 d were analyzed by PCR together with A1-H.1 and ΔNBPXa controls.

The difference between adhesion and invasion of merozoites was also measured using an immunofluorescence assay. Synchronous *P. knowlesi* A1-H.1 or *ΔNBPXa* schizonts were added to triplicate cultures at 2% parasitemia with cynomolgus or human RBC, and after 3 h thin blood films were prepared for immunofluorescence staining. The number of free merozoites ($MSP1_{19}$-positive, $MSP1_{33}$-positive, no contact with RBC), attached merozoites ($MSP1_{19}$-positive, $MSP1_{33}$-positive, in contact with RBCs), rings ($MSP1_{19}$-positive, $MSP1_{33}$-negative, within RBCs), and RBCs were counted. A minimum of 1,000 RBCs was examined.

DNA Constructs and PCR. The DNA construct (NBPXa_1XKO) for targeted disruption of *NBPXa* (GeneDB ID PKNH_1472300) via single–cross-over homologous recombination was adapted from a precursor to the $PkconGFP_{ep}$ vector (21) and used a 1.2-kb region of the 5′ end of the *NBPXa* gene to target the NBPXa locus, with linearization for transfection using the BsiWI site situated in the *NBPXa* fragment.

Transfection, Cloning, and Genotyping. Parasites were transfected with NBPXa_1XKO or with plasmid PkconGFPp230p (21) as a positive control, and fresh human or cynomolgus RBCs were added every 4 d until parasites were visible. Parasites were cloned by limiting dilution, a process requiring 9 or 14 d in cynomolgus or human RBCs, respectively, and then were expanded for genotypic and phenotypic analysis. Transfected parasites were analyzed by diagnostic PCR with two different primer sets. The first was designed to amplify a 1,478-bp fragment only from the intact wild-type *NBPXa* locus (ol1 and ol2), and the second was designed to amplify a 1,306-bp fragment after correct integration of NBPXa_1XKO (ol1 and ol3). PCR reactions were carried out using GoTaq Master Mix (Promega) using the following cycle conditions: 3 min at 96 °C, then 30 cycles of 25 s at 96 °C, 25 s at 52 °C, and 2 min at 64 °C, and a final extension of 5 min at 64 °C.

ACKNOWLEDGMENTS. This work was supported by the Francis Crick Institute, which receives its core funding from Cancer Research UK, the UK Medical Research Council (MRC), and the Wellcome Trust. The work was further supported by the UK MRC (Grants U117532063 and U117532067), the European Community's Seventh Framework Programme under Grant Agreement 242095 (EviMalar), an MRC Career Development Award (to R.W.M.) jointly funded by the UK MRC and Department for International Development, and the faculty baseline fund (BRF) from the King Abdullah University of Science and Technology (to A.P.). A.A. and Y.L.L. were funded by a UM High Impact Research (HIR) Grant UM-MOHE (UM.C/HIR/MOHE/MED/16) from the Ministry of Higher Education Malaysia.

1. Lee K-S, et al. (2011) *Plasmodium knowlesi*: Reservoir hosts and tracking the emergence in humans and macaques. *PLoS Pathog* 7(4):e1002015–e1002015.
2. Singh B, et al. (2004) A large focus of naturally acquired *Plasmodium knowlesi* infections in human beings. *Lancet* 363(9414):1017–1024.
3. William T, et al. (2013) Increasing incidence of *Plasmodium knowlesi* malaria following control of *P. falciparum* and *P. vivax* Malaria in Sabah, Malaysia. *PLoS Negl Trop Dis* 7(1):e2026–e2026.
4. Yusof R, et al. (2014) High proportion of knowlesi malaria in recent malaria cases in Malaysia. *Malar J* 13(1):168.
5. Cowman AF, Crabb BS (2006) Invasion of red blood cells by malaria parasites. *Cell* 124(4):755–766.
6. Miller LH, Mason SJ, Dvorak JA, McGinniss MH, Rothman IK (1975) Erythrocyte receptors for (*Plasmodium knowlesi*) malaria: Duffy blood group determinants. *Science* 189(4202):561–563.
7. Ahmed AM, et al. (2014) Disease progression in *Plasmodium knowlesi* malaria is linked to variation in invasion gene family members. *PLoS Negl Trop Dis* 8(8):e3086.
8. Wanaguru M, Liu W, Hahn BH, Rayner JC, Wright GJ (2013) RH5-Basigin interaction plays a major role in the host tropism of *Plasmodium falciparum*. *Proc Natl Acad Sci USA* 110(51):20735–20740.
9. Tham W-H, Healer J, Cowman AF (2012) Erythrocyte and reticulocyte binding-like proteins of *Plasmodium falciparum*. *Trends Parasitol* 28(1):23–30.
10. Galinski MR, Medina CC, Ingravallo P, Barnwell JW (1992) A reticulocyte-binding protein complex of *Plasmodium vivax* merozoites. *Cell* 69(7):1213–1226.
11. Okenu DM, et al. (2005) The reticulocyte binding proteins of *Plasmodium cynomolgi*: A model system for studies of *P. vivax*. *Mol Biochem Parasitol* 143(1):116–120.
12. Ogun SA, et al. (2011) Targeted disruption of py235ebp-1: Invasion of erythrocytes by *Plasmodium yoelii* using an alternative Py235 erythrocyte binding protein. *PLoS Pathog* 7(2):e1001288.
13. Rayner JC, Galinski MR, Ingravallo P, Barnwell JW (2000) Two Plasmodium falciparum genes express merozoite proteins that are related to *Plasmodium vivax* and *Plasmodium yoelii* adhesive proteins involved in host cell selection and invasion. *Proc Natl Acad Sci USA* 97(17):9648–9653.
14. Triglia T, et al. (2001) Identification of proteins from *Plasmodium falciparum* that are homologous to reticulocyte binding proteins in *Plasmodium vivax*. *Infect Immun* 69(2):1084–1092.
15. Meyer EVS, et al. (2009) The reticulocyte binding-like proteins of *P. knowlesi* locate to the micronemes of merozoites and define two new members of this invasion ligand family. *Mol Biochem Parasitol* 165(2):111–121.
16. Semenya AA, Tran TM, Meyer EV, Barnwell JW, Galinski MR (2012) Two functional reticulocyte binding-like (RBL) invasion ligands of zoonotic *Plasmodium knowlesi* exhibit differential adhesion to monkey and human erythrocytes. *Malar J* 11:228.
17. Baum J, et al. (2009) Reticulocyte-binding protein homologue 5—An essential adhesin involved in invasion of human erythrocytes by *Plasmodium falciparum*. *Int J Parasitol* 39(3):371–380.
18. Adams JH, et al. (1990) The Duffy receptor family of *Plasmodium knowlesi* is located within the micronemes of invasive malaria merozoites. *Cell* 63(1):141–153.
19. Singh AP, et al. (2005) Targeted deletion of *Plasmodium knowlesi* Duffy binding protein confirms its role in junction formation during invasion. *Mol Microbiol* 55(6):1925–1934.
20. Kocken CHM, et al. (2002) *Plasmodium knowlesi* provides a rapid in vitro and in vivo transfection system that enables double-crossover gene knockout studies. *Infect Immun* 70(2):655–660.
21. Moon RW, et al. (2013) Adaptation of the genetically tractable malaria pathogen *Plasmodium knowlesi* to continuous culture in human erythrocytes. *Proc Natl Acad Sci USA* 110(2):531–536.
22. Lim C, et al. (2013) Expansion of host cellular niche can drive adaptation of a zoonotic malaria parasite to humans. *Nat Commun* 4:1638.
23. Chin W, Contacos PG, Coatney GR, Kimball HR (1965) A naturally acquired quotidian-type malaria in man transferable to monkeys. *Science* 149(3686):865.
24. Pain A, et al. (2008) The genome of the simian and human malaria parasite *Plasmodium knowlesi*. *Nature* 455(7214):799–803.
25. Assefa S, et al. (2015) Population genomic structure and adaptation in the zoonotic malaria parasite *Plasmodium knowlesi*. *Proc Natl Acad Sci USA* 112(42):13027–13032.
26. Theron M, Hesketh RL, Subramanian S, Rayner JC (2010) An adaptable two-color flow cytometric assay to quantitate the invasion of erythrocytes by *Plasmodium falciparum* parasites. *Cytometry A* 77(11):1067–1074.
27. Blackman MJ, et al. (1996) *Plasmodium knowlesi*: Secondary processing of the malaria merozoite surface protein-1. *Exp Parasitol* 83(2):229–239.
28. Jun G, et al. (2009) Factors affecting reticulocyte enrichment by density gradient ultracentrifugation. *Acta Haematol* 121(4):207–211.
29. Sorette MP, Shiffer K, Clark MR (1992) Improved isolation of normal human reticulocytes via exploitation of chloride-dependent potassium transport. *Blood* 80(1):249–254.
30. Nair S, et al. (2008) Adaptive copy number evolution in malaria parasites. *PLoS Genet* 4(10):e1000243.
31. Satkoski Trask JA, et al. (2013) Single-nucleotide polymorphisms reveal patterns of allele sharing across the species boundary between rhesus (*Macaca mulatta*) and cynomolgus (*M. fascicularis*) macaques. *Am J Primatol* 75(2):135–144.
32. Carlton JM, Das A, Escalante AA (2013) Genomics, population genetics and evolutionary history of *Plasmodium vivax*. *Adv Parasitol* 81:203–222.
33. Adams JH, et al. (1992) A family of erythrocyte binding proteins of malaria parasites. *Proc Natl Acad Sci USA* 89(15):7085–7089.
34. Miller LH, Mason SJ, Clyde DF, McGinniss MH (1976) The resistance factor to *Plasmodium vivax* in blacks. The Duffy-blood-group genotype, FyFy. *N Engl J Med* 295(6):302–304.
35. Carlton JM, et al. (2008) Comparative genomics of the neglected human malaria parasite *Plasmodium vivax*. *Nature* 455(7214):757–763.
36. Hester J, et al. (2013) De novo assembly of a field isolate genome reveals novel *Plasmodium vivax* erythrocyte invasion genes. *PLoS Negl Trop Dis* 7(12):e2569.
37. Tachibana S, et al. (2012) *Plasmodium cynomolgi* genome sequences provide insight into *Plasmodium vivax* and the monkey malaria clade. *Nat Genet* 44(9):1051–1055.
38. Singh S, Alam MM, Pal-Bhowmick I, Brzostowski JA, Chitnis CE (2010) Distinct external signals trigger sequential release of apical organelles during erythrocyte invasion by malaria parasites. *PLoS Pathog* 6(2):e1000746.
39. Ranjan A, Chitnis CE (1999) Mapping regions containing binding residues within functional domains of *Plasmodium vivax* and *Plasmodium knowlesi* erythrocyte-binding proteins. *Proc Natl Acad Sci USA* 96(24):14067–14072.
40. Pinheiro MM, et al. (2015) *Plasmodium knowlesi* genome sequences from clinical isolates reveal extensive genomic dimorphism. *PLoS One* 10(4):e0121303.
41. Otto TD, Dillon GP, Degrave WS, Berriman M (2011) RATT: Rapid Annotation Transfer Tool. *Nucleic Acids Res* 39(9):e57.
42. Boeva V, et al. (2012) Control-FREEC: A tool for assessing copy number and allelic content using next-generation sequencing data. *Bioinformatics* 28(3):423–425.
43. Abyzov A, Urban AE, Snyder M, Gerstein M (2011) CNVnator: An approach to discover, genotype, and characterize typical and atypical CNVs from family and population genome sequencing. *Genome Res* 21(6):974–984.
44. Smith SD, Kawash JK, Grigoriev A (2015) GROM-RD: Resolving genomic biases to improve read depth detection of copy number variants. *PeerJ PrePrints* 3:e836.