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

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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 cyonmolus 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.

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.


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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. 1A). 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. 1A 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. 1B and Fig. S1). In A1-O and A1-H, this region encompasses 12 genes (Table S2), including PKNH1 (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 DBP\( \alpha \), DBP1, DBP\( \gamma \), and NBXPb 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. 2 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 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. 2A). Further examination of the cultures showed no evidence of defects in subsequent intracellular growth (Fig. 2B 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 line clearly did not reduce its invasion efficiency for cynomolgus cells. We therefore reasoned that its loss may have
caused the shift in host cell selectivity through reduced invasion efficiency for human RBCs. The ability of the A1-H.1 line to grow intracellularly in cynomolgus RBCs offered a unique opportunity to test this hypothesis. No other clear relationship between genotype and adaptation to culture was established.

The \textit{NBPXa} Gene Can Be Disrupted in A1-H.1 Parasites Grown in Cynomolgus But Not in Human RBCs. To analyze the role of \textit{NBPXa}, A1-H.1 parasites were transfected with a gene-disruption construct (Fig. 3A) 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 \textit{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. 3B). Thus, the \textit{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 \(\Delta\text{NBPXa}\) cloned lines were derived from A1-H.1 parasite transfections in cynomolgus RBCs and were used for subsequent phenotypic analysis.

\textit{NBPXa} Is Unnecessary for Invasion of Cynomolgus RBCs but Is Crucial for Invasion of Human RBCs. We examined the ability of the \(\Delta\text{NBPXa}\) clones to invade and grow within either human or cynomolgus RBCs in the 24-h invasion assay. Disruption of \textit{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. 4A and Fig. S3). The defect in human RBC invasion caused by the loss of the \textit{NBPXa} gene was severe and prevented multiplicative growth, but a low frequency of successful invasions was detected for both the A1-C.1 and \(\Delta\text{NBPXa}\) 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 \(\Delta\text{NBPXa}\) and A1C.1 parasites was able to infect human RBCs. Parasitemia measurements at 48 h showed that the \(\Delta\text{NBPXa}\) human RBC invasion phenotype was expressed in subsequent cycles, and the parasite could not undergo multiplicative growth in human RBCs (Fig. 4B and C). Extended culture of \(\Delta\text{NBPXa}\) 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 \textit{NBPXa} disruptant construct (Fig. S3). These data demonstrate the severity of the \(\Delta\text{NBPXa}\) phenotype, accounting for both the inability to disrupt the \textit{NBPXa} gene and the severely reduced growth of the A1-C.1 line in human RBCs.

Further analysis showed that the \(\Delta\text{NBPXa}\) 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. 4B 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 \(\Delta\text{NBPXa}\) parasites appeared to be extracellular merozoites that had attached to but had not invaded RBCs (Fig. 4D 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\textsuperscript{rop}-positive only) from those that had attached but failed to invade, (MSP1\textsubscript{33} and MSP1\textsubscript{19} double positive) (Fig. 5A). Although 92% of A1-H.1 merozoites successfully formed rings in human RBCs, 76% of \(\Delta\text{NBPXa}\) merozoites attacked but failed to invade human RBCs (Fig. 5B). In contrast, about 90% of merozoites from both lines successfully formed rings in cynomolgus RBCs (Fig. 5B). These results show that \textit{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 \(\Delta\text{NBPXa}\) parasites. This analysis did not identify any significantly differentially expressed genes.

\textbf{Deletion of \textit{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 \(\Delta\text{NBPXa}\) and A1-C.1 lines invaded a small percentage of human RBCs raised the possibility that the loss of \textit{NBPXa} function restricted the parasite to invasion of reticulocytes, which typically constitute 0.5–2% of total RBCs. To investigate this possibility, we

\begin{figure*}[h]
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\caption{Disruption of \textit{NBPXa} can be achieved when parasites are grown in macaque RBCs but not in human RBCs. (A) Disruption of \textit{NBPXa} by the introduction of linearized p\textit{NBPXa}_1XKO into the genome via integration at the 5'-end of the \textit{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 T1.2) confirmed correct integration only for parasites grown in cynomolgus RBCs. (Upper) Amplification of a product from wild-type \textit{NBPXa} (primers o1 and o2). (Lower) Amplification of a product from a species that can be produced only following integration (primers o1 and o3).}
\end{figure*}
Fig. 4. Disruption of NBPXa prevents multiplicative growth in human RBCs through impaired invasion. (A) Growth of A1-C.1, A1-H.1, and ΔNBPXa 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, ΔNBPXa showed no significant difference in growth in cynomolgus but a significant reduction in human RBCs (*P = 0.00481). The growth of ΔNBPXa 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 ΔNBPXa (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.)

ΔNBPXa 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) 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.

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 uncloined 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 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. ΔNBPXa 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-MSP1α (green), anti-MSP1β (red), and a merge of both MSP1 antibodies with anti-human RBC (magenta) and DAPI (blue). MSP1 is processed during invasion, with MSP1α being shed and MSP1β retained, distinguishing external attached merozoites (both MSP1α- and MSP1β-positive) from those that had successfully invaded to form rings (only MSP1β-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 invasion 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 NBPxβ expressed on cells binds cynomolgus RBCs four times more efficiently 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 parasites use an orthologous DBP binding 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* NBPxα 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 of the 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 NBPxα 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. Phylogenetically distinct species within 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 adhesion requirements enables the generation of lines with gene knockouts for important parasite adhesins such as NBPxα, providing a powerful and unique opportunity to investigate their precise role in red cell invasion.

The adhesion repertoire is smaller in *P. knowlesi* than in many malaria parasites (9, 15, 18), and the loss of either DBPα (6, 19) or NBPxα creates a critical block in the invasion of human RBCs, perhaps because the paralogues cannot bind human RBCs. For example, although PKNBPxα and DBPα proteins bind to both human and macaque RBCs, DBPβ, DBPγ, and NBPxβ 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/NBPxα 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 and useful vaccine development tool. Vaccination against one of these molecules may be an important strategy for control of *P. knowlesi* malaria, given the presence of a primate reservoir in Southeast Asia.

NBPxα 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 NBPxβ, which had been found not to bind to human RBCs, has also been linked to disease severity in humans (7, 16). The role of NBPxβ and variants of both *P. knowlesi* 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 Animal (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 Megraud (National Institutes of Health, Bethesda, MD), following passage in vivo in rhesus macaque of the *P. knowlesi* H strain derived from a clinical infection (23). A1-O is a nonclonal cultural 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); P. vivax reference genome Illumina library; P. knowlesi reference genome Illumina library; 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 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. Parasitism 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-carbazole (2,7-dichloro-9,9-dimethylacridin-2-one) succinimidyl ester (DDAO-S) (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).

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The DNA construct (NPBXa_1XKO) for targeted disruption of NPBXa (GeneDB ID PKNH_14723000) via single-cross-over homologous recombination was adapted from a precursor to the PkcnGP1r vector (21) and used a 1.2-kb region of the 5′-end of the NPBXa gene to target the NPBXa npx, with linearization for transfection using the BsuRI site situated in the NPBXa fragment.

Transfection, Cloning, and Genotyping. Parasites were transfected with NPBXa, TXKO or with plasmid PkcnGP2p230p (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 until parasites were visible. The DNA construct (NPBXa_1XKO) for targeted disruption of NPBXa (GeneDB ID PKNH_14723000) via single-cross-over homologous recombination was adapted from a precursor to the PkcnGP1r vector (21) and used a 1.2-kb region of the 5′-end of the NPBXa gene to target the NPBXa npx, with linearization for transfection using the BsuRI site situated in the NPBXa fragment.

Then the cells were pelleted, and Giemsa-stained thin blood films were prepared. To attempt to readapt ΔNPBXa 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 ΔNPBXa controls.

The difference between adhesion and invasion of merozoites was also measured using an immunofluorescence assay. Symaphonous P. knowlesi A1-H.1 or ΔNPBXa schizonts were added to triplicate cultures in 2% para-

DNA Constructs and PCR. The DNA construct (NPBXa_1XKO) for targeted disruption of NPBXa (GeneDB ID PKNH_14723000) via single-cross-over homologous recombination was adapted from a precursor to the PkcnGP1r vector (21) and used a 1.2-kb region of the 5′-end of the NPBXa gene to target the NPBXa npx, with linearization for transfection using the BsuRI site situated in the NPBXa fragment.

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