Plasmodium P36 determines host cell receptor usage during sporozoite invasion

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ABSTRACT

*Plasmodium* sporozoites, the mosquito-transmitted forms of the malaria parasite, first infect the liver for an initial round of replication before the emergence of pathogenic blood stages. Sporozoites represent attractive targets for antimalarial preventive strategies, yet the mechanisms of parasite entry into hepatocytes remain poorly understood. Here we show that the two main species causing malaria in humans, *Plasmodium falciparum* and *Plasmodium vivax*, rely on two distinct host cell surface proteins, CD81 and the Scavenger Receptor BI (SR-BI), respectively, to infect hepatocytes. By contrast, CD81 and SR-BI fulfil redundant functions during infection by the rodent parasite *P. berghei*. Genetic analysis of sporozoite factors reveals the 6-cysteine domain protein P36 as a major parasite determinant of host cell receptor usage. Our data provide molecular insights into the invasion pathways used by different malaria parasites to infect hepatocytes, and establish a functional link between a sporozoite putative ligand and host cell receptors.
INTRODUCTION

Hepatocytes are the main cellular component of the liver and the first replication niche for the malaria-causing parasite *Plasmodium*. Malaria begins with the inoculation of sporozoites into the host skin by infected *Anopheles* mosquitoes. Sporozoites rapidly migrate to the liver and actively invade hepatocytes by forming a specialized compartment, the parasitophorous vacuole (PV), where they differentiate into thousands of merozoites (1). Once released in the blood, merozoites invade and multiply inside erythrocytes, causing the malaria disease.

Under natural transmission conditions, infection of the liver is an essential, initial and clinically silent phase of malaria, and therefore constitutes an ideal target for prophylactic intervention strategies. However, the molecular mechanisms underlying *Plasmodium* sporozoite entry into hepatocytes remain poorly understood. Highly sulphated proteoglycans in the liver sinusoids are known to bind the circumsporozoite protein, which covers the parasite surface, and contribute to the homing and activation of sporozoites (2,3). Subsequent molecular interactions leading to sporozoite entry into hepatocytes have not been identified yet. Several parasite proteins have been implicated, such as the thrombospondin related anonymous protein (TRAP) (4), the apical membrane antigen 1 (AMA-1) (5), or the 6-cysteine domain proteins P52 and P36 (6–10), however their role during sporozoite invasion remains unclear (11).

Our previous work highlighted the central role of the host tetraspanin CD81, one of the receptors for the hepatitis C virus (HCV) (12), during *Plasmodium* liver infection (13). CD81 is an essential host entry factor for human-infecting *P. falciparum* and rodent-infecting *P. yoelii* sporozoites (13,14). CD81 acts at an early step of invasion, possibly by providing signals that trigger the secretion of rhoptries, a set of apical organelles involved in PV formation (15). Whereas CD81 binds the HCV E2 envelope protein (12), there is no evidence for such a direct interaction between CD81 and *Plasmodium* sporozoites (13). Rather, we proposed that CD81 acts indirectly, possibly by regulating an as yet unidentified receptor for sporozoites within cholesterol-dependent tetraspanin-enriched microdomains (16,17).
Intriguingly, the rodent malaria parasite *P. berghei* can infect cells lacking CD81 (13,18), however the molecular basis of this alternative entry pathway was until now totally unknown.

Another hepatocyte surface protein, the scavenger receptor BI (SR-BI), was shown to play a dual role during malaria liver infection, first in promoting parasite entry and subsequently its development inside hepatocytes (19,20). However, the contribution of SR-BI during parasite entry is still unclear. SR-BI, which is also a HCV entry factor (21,22), binds high-density lipoproteins with high affinity and mediates selective cellular uptake of cholesteryl esters (23). Yalaoui et al. reported that SR-BI is involved indirectly during *P. yoelii* sporozoite invasion, by regulating the levels of membrane cholesterol and the expression of CD81 and its localization in tetraspanin-enriched microdomains (19). In another study, Rodrigues et al. observed a reduction of *P. berghei* invasion of Huh-7 cells upon SR-BI inhibition (20). Since CD81 is not required for *P. berghei* sporozoite entry into Huh-7 cells (18), these results suggested a CD81-independent role for SR-BI. More recently, Foquet et al. showed that anti-CD81 but not anti-SR-BI antibodies inhibit *P. falciparum* sporozoite infection in humanized mice engrafted with human hepatocytes (24), questioning the role of SR-BI during *P. falciparum* infection.

These conflicting results prompted us to revisit the contribution of SR-BI during *P. falciparum*, *P. yoelii* and *P. berghei* sporozoite infections. For the first time, we also explored the role of CD81 and SR-BI during hepatocyte infection by *P. vivax*, a widely distributed yet highly neglected cause of malaria in humans, for which the contribution of hepatocyte surface receptors has not been investigated to date.

Here, we show that SR-BI is an important entry factor for *P. vivax* but not for *P. falciparum* or *P. yoelii* sporozoites. Remarkably, SR-BI and CD81 fulfil redundant functions during host cell invasion by *P. berghei* sporozoites, which can use one or the other molecule. We further investigated parasite determinants associated with host cell receptor usage. We show that genetic depletion of P52 and P36, two members of the *Plasmodium* 6-cysteine domain protein family, abrogates sporozoite productive invasion and mimics the inhibition of CD81 and/or SR-BI entry pathways, in both *P. berghei* and *P. yoelii*. Finally, we identify P36
as the molecular driver of *P. berghei* sporozoite entry via SR-BI. Our data, by revealing a functional link between parasite and host cell entry factors, pave the way towards the identification of ligand-receptor interactions mediating *Plasmodium* infection of hepatocytes, and open novel perspectives for preventive and therapeutic approaches.
RESULTS

Antibodies against SR-BI inhibit *P. vivax* but not *P. falciparum* or *P. yoelii* sporozoite infection

To evaluate the contribution of CD81 and SR-BI during *P. vivax* infection, we tested the effects of neutralizing CD81- and/or SR-BI-specific antibodies on *P. vivax* sporozoite infection in primary human hepatocyte cultures (25). A monoclonal antibody (mAb) against the main extracellular domain of CD81, previously shown to inhibit *P. falciparum* sporozoite infection (13), had no effect on the number of *P. vivax*-infected cells *in vitro* (Figure 1A). In sharp contrast, a mouse mAb specific for SR-BI (26) greatly reduced infection, with no additive effect of anti-CD81 antibodies (Figure 1A). The same inhibitory effect was observed using polyclonal anti-SR-BI antibodies (Figure 1B). We performed the same experiments with *P. falciparum* sporozoites and found that anti-CD81 but not anti-SR-BI antibodies inhibit *P. falciparum* infection *in vitro* (Figure 1C), in agreement with the *in vivo* data from Foquet *et al* (24). These data strongly suggest that *P. vivax* and *P. falciparum* sporozoites use distinct entry pathways to infect hepatocytes, reminiscent of the differences between *P. berghei* and *P. yoelii* (13).

Two distinct populations of *P. vivax* exo-erythrocytic forms (EEFs) could be distinguished in the infected cultures, large EEFs representing replicating schizonts, and small EEFs that may correspond to hypnozoites (27) (Figure 1D). Anti-SR-BI antibodies reduced the numbers of large and small EEFs to the same extent (Figure 1E), suggesting an effect on sporozoite invasion rather than on parasite intracellular development.

SR-BI is required for productive invasion of CD81-null cells by *P. berghei* sporozoites

In order to investigate in more details the role of SR-BI during sporozoite entry, we used the more tractable rodent malaria parasite *P. berghei*. Indeed, *P. berghei* sporozoites readily infect HepG2 cells, which lack CD81 (28,29) but express high levels of SR-BI (Figure 2-figure supplement 1), raising the possibility that this rodent parasite uses a SR-BI route to infect CD81-null cells. To test this hypothesis, *P. berghei* sporozoites constitutively
expressing GFP (PbGFP) (30) were incubated with HepG2 cells in the presence of increasing concentrations of polyclonal anti-SR-BI rabbit antibodies. We observed a dramatic and dose-dependent reduction of the number of EEF-infected cells induced by anti-SR-BI antibodies (Figure 2A). Quantification of host cell invasion by FACS demonstrated that the rabbit anti-SR-BI antibodies block infection at the invasion step (Figure 2B). Similar results were obtained with polyclonal rat antibodies and a mouse mAb directed against human SR-BI (Figure 2-figure supplement 2).

As a complementary approach, we used small interfering RNA (siRNA) to specifically knockdown SR-BI expression in HepG2 cells (Figure 2D). SR-BI silencing caused a dramatic reduction of the number of EEF-infected cells (Figure 2E), but had no significant effect on the intracellular development of the few invaded parasites (Figure 2F). *Plasmodium* sporozoites migrate through several cells before invading a final one inside a PV (31). Sporozoite cell traversal was increased in SR-BI-depleted HepG2 cells, as compared to the control (Figure 2-figure supplement 3). This is likely due to the robust cell traversal activity of *P. berghei* sporozoites, which continue to migrate through cells when productive invasion is impaired. Altogether, these data establish that SR-BI is a major entry factor for *P. berghei* sporozoites in CD81-null HepG2 cells.

**CD81 and SR-BI play redundant roles during *P. berghei* sporozoite invasion**

We next tested whether the presence of CD81 would affect SR-BI function during *P. berghei* sporozoite infection. We monitored invasion and replication of *P. berghei* sporozoites in HepG2 cells genetically engineered to express CD81 (HepG2/CD81) (Figure 2-figure supplement 1) (14), in the presence of anti-SR-BI and/or anti-CD81 antibodies. Strikingly, unlike in CD81-null HepG2 cells, anti-SR-BI antibodies had no inhibitory effect on *P. berghei* infection in HepG2/CD81 cells (Figure 3A). Remarkably, whilst neither anti-SR-BI nor anti-CD81 antibodies alone had any significant impact on invasion (Figure 3A) or parasite intracellular development (Figure 3-figure supplement 1), the combination of CD81 and SR-BI antibodies markedly reduced the number of infected cells (Figure 3A). Similarly,
siRNA-mediated silencing of either CD81 or SR-BI alone had no effect on infection, whereas simultaneous silencing of both factors greatly reduced infection (*Figure 3B*).

Blocking both CD81 and SR-BI was associated with an increase in cell traversal activity (*Figure 3-figure supplement 1*), suggesting that the concomitant neutralization of the two host factors prevented commitment to productive invasion. To directly test this hypothesis, we analysed the invasion kinetics of PbGFP sporozoites in HepG2/CD81 cells, in the presence of anti-SR-BI and/or anti-CD81 neutralizing antibodies. We have shown that *in vitro* sporozoite invasion follows a two-step kinetics (32), with initially low invasion rates at early time points, reflecting cell traversal activity, followed by a second phase of productive invasion associated with PV formation. In HepG2/CD81 cells, the invasion kinetics of *P. berghei* sporozoites in the presence of anti-SR-BI or anti-CD81 specific antibodies were comparable to those of the control without antibody (*Figure 3C*). In sharp contrast, blocking both CD81 and SR-BI simultaneously suppressed the second phase of productive invasion (*Figure 3C*). Based on these results, we conclude that SR-BI and CD81 are involved in the commitment to productive entry.

*P. berghei* sporozoites infect mouse hepatocytic Hepa1-6 cells via a CD81-dependent pathway, as shown by efficient inhibition of infection by CD81 specific antibodies or siRNA (18). Interestingly, we failed to detect SR-BI in Hepa1-6 cells (*Figure 3D*), providing a plausible explanation as to why CD81 is required for *P. berghei* infection in this model. We tested whether ectopic expression of SR-BI would rescue *P. berghei* infection of Hepa1-6 cells upon silencing of endogenous murine CD81 by siRNA. CD81-silenced Hepa1-6 cells were transiently transfected with plasmids encoding human SR-BI or CD81, before exposure to *P. berghei* sporozoites. The number of infected cells was greatly reduced in CD81-silenced cells as compared to control non-silenced cells (*Figure 3E*), in agreement with our previous observations (18). Remarkably, transfection of either human CD81 or human SR-BI was sufficient to rescue infection in CD81-silenced cells (*Figure 3E*), demonstrating that the two receptors can independently perform the same function to support *P. berghei* infection.
Collectively, our data provide direct evidence that CD81 and SR-BI play redundant roles during productive invasion of hepatocytic cells by *P. berghei* sporozoites.

**P. yoelii** sporozoites require host CD81 but not SR-BI for infection

We next investigated the contribution of SR-BI to *P. yoelii* sporozoite infection in HepG2/CD81 cells. *P. yoelii* infection was dramatically reduced by anti-CD81 antibodies, consistent with our previous work (14), but strikingly was not affected by anti-SR-BI antibodies (*Figure 4A*). In addition, *P. yoelii* infection was not affected by siRNA-mediated silencing of SR-BI, but was almost abolished upon knockdown of CD81 (*Figure 4B*). These results indicate that CD81 but not SR-BI plays a central role during *P. yoelii* sporozoite invasion in HepG2/CD81 cells, similarly to *P. falciparum* in human hepatocytes.

**The 6-cysteine domain proteins P52 and P36 are required for sporozoite productive invasion irrespective of the host cell entry pathway**

The data above show that *P. berghei* sporozoites can use either CD81 or SR-BI to infect hepatocytic cells, whereas *P. yoelii* utilizes CD81 but not SR-BI, suggesting that one or several *P. berghei* factors may be specifically associated with SR-BI usage. Among potential candidate parasite factors involved in sporozoite entry, we focused on the 6-cysteine domain proteins P52 and P36, two micronemal proteins of unknown function previously implicated during liver infection (6–10,33,34). To facilitate monitoring of the role of P52 and P36 during host cell invasion, we used a Gene Out Marker Out (GOMO) strategy (30) to generate highly fluorescent *p52/p36*-knockout parasite lines in *P. berghei* (*Figure 5-figure supplement 1*) and *P. yoelii* (*Figure 5-figure supplement 2*). Pure populations of GFP-expressing, drug selectable marker-free PbΔ*p52/p36* and PyΔ*p52/p36* blood stage parasites were obtained and transmitted to mosquitoes in order to produce sporozoites.

Analysis of the kinetics of PbΔ*p52/p36* sporozoite invasion by FACS, in comparison to control PbGFP sporozoites, revealed that genetic ablation of *p52/p36* abrogates sporozoite productive invasion of HepG2 (SR-BI-dependent entry pathway) and Hepa1-6...
cells (CD81-dependent entry pathway) (Figure 5A and 5B). PbΔp52/p36 sporozoite invasion followed similar kinetics to those observed for PbGFP sporozoites upon blockage of SR-BI or CD81, respectively, and was not modified by addition of anti-SRBI or anti-CD81 antibodies. Using antibodies specific for UIS4, a marker of the PV membrane (PVM) that specifically labels productive vacuoles (32,35), we confirmed that PbGFP but not PbΔp52/p36 parasites could form replicative PVs, in both HepG2 and HepG2/CD81 cells (Figure 5C). In both cell types, only very low numbers of EEFs were observed with PbΔp52/p36 parasites (Figure 5D), all of which were seemingly intranuclear and lacked a UIS4-labeled PVM (Figure 5E).

We have shown before that intranuclear EEFs result from cell traversal events (14). Altogether these data demonstrate that PbΔp52/p36 sporozoites fail to productively invade host cells, irrespective of the entry route. Similar results were obtained with a P. berghei Δp36 single knockout line using the GOMO strategy (Figure 5-figure supplement 3). PbΔp36 sporozoites did not invade HepG2 cells or HepG2/CD81 cells, reproducing a similar phenotype as PbGFP parasites in the presence of anti-CD81 and anti-SR-BI neutralizing antibodies (Figure 5-figure supplement 4).

We then examined the kinetics of P. yoelii Δp52/p36 sporozoite invasion, in comparison to those of PyGFP sporozoites, in HepG2/CD81 versus HepG2 cells. PyΔp52/p36 sporozoites showed a lack of productive invasion in HepG2/CD81 cells, reproducing the invasion kinetics of PyGFP in the CD81-null HepG2 cells (Figure 5F). The PyΔp52/p36 mutant failed to form PV in HepG2/CD81 cells (Figure 5G), where only intranuclear EEFs lacking a UIS4-labeled PVM were observed, similarly to the control PyGFP parasites in HepG2 cells (Figure 5H and 5I).

Altogether, these results reveal that P52 and/or P36 are required for sporozoite productive invasion, in both P. berghei and P. yoelii, irrespective of the entry route.

P36 is a key determinant of host cell receptor usage during sporozoite invasion

We further sought to investigate whether P52 and/or P36 proteins contribute to the selective usage of host cell receptors by different sporozoite species. We designed a trans-
species genetic complementation strategy in which copies of *P. berghei* (Pb), *P. yoelii* (Py), *P. falciparum* (Pf) or *P. vivax* (Pv) P52 and P36 were introduced in the Δp52/p36 parasites. For this purpose, we used centromeric plasmid constructs for stable expression of the transgenes from episomes (36). Complementing PbΔp52/p36 sporozoites with PbP52 and PbP36 restored sporozoite infectivity to both HepG2 and HepG2/CD81 cells (*Figure 6A*), where the parasite formed UIS4-positive vacuoles (*Figure 6B, 6C and 6D*), confirming that genetic complementation was efficient. Remarkably, complementation of PbΔp52/p36 with PyP52 and PyP36 restored infection in HepG2/CD81 but not in HepG2 cells (*Figure 6A*), where only low numbers of UIS4-negative intranuclear EEFs were observed (*Figure 6B and 6D*). Thus, the concomitant replacement of PbP52 and PbP36 by their *P. yoelii* counterparts reproduced a *P. yoelii*-like invasion phenotype in chimeric *P. berghei* sporozoites, indicating that P52 and/or P36 contribute to the selective usage of a CD81-independent entry pathway in *P. berghei* sporozoites. Complementation of PbΔp52/p36 parasites with either *P. falciparum* or *P. vivax* P52 and P36 coding sequences did not restore infectivity of transgenic sporozoites, not only in HepG2 and HepG2/CD81 cells (*Figure 6-figure supplement 1A*), but also in primary human hepatocytes, the most permissive cellular system for human malaria sporozoites *in vitro* (*Figure 6-figure supplement 1B*). Hence it was not possible using this approach to assess the function of *P. falciparum* or *P. vivax* P52 and P36 in transgenic *P. berghei* sporozoites.

We next dissected the individual contribution of P52 and P36 by complementing PbΔp52/p36 parasites with mixed combinations of either PyP52 and PbP36 or PbP52 and PyP36. This approach revealed that P36 determines the ability of *P. berghei* sporozoites to enter cells via a CD81-independent route. Indeed, PbΔp52/p36 complemented with PyP52 and PbP36 infected both HepG2/CD81 and HepG2 cells (*Figure 6A*), forming UIS4-labeled PVs in both cell types (*Figure 6B, 6C and 6D*). P52 therefore is not responsible for the phenotypic difference between *P. berghei* and *P. yoelii*. In sharp contrast, complementation of PbΔp52/p36 with PbP52 and PyP36 restored sporozoite infectivity to HepG2/CD81 but not HepG2 cells, thus reproducing a *P. yoelii*-like invasion phenotype (*Figure 6A, 6B and 6D*).
In reciprocal experiments, we analysed whether expression of PbP36 would be sufficient to allow *P. yoelii* sporozoites to invade CD81-null cells. For this purpose, we performed genetic complementation experiments in PyΔp52/p36 parasites, using the same constructs employed with the *P. berghei* mutant. Strikingly, whilst HepG2 cells are normally refractory to *P. yoelii* productive invasion, complementation with *P. berghei* P52 and P36 protein was sufficient to confer chimeric *P. yoelii* mutants the capacity to infect HepG2 cells (*Figure 7A*). Most importantly, PyΔp52/p36 parasites complemented with PyP52 and PbP36 infected both HepG2 and HepG2/CD81 cells (*Figure 7A*). In particular, PyΔp52/p36 parasites expressing PbP36 became capable of forming UIS4-positive PVs in HepG2 cells (*Figure 7B*). Thus, the transgenic expression of PbP36 appears to be sufficient to recapitulate a *P. berghei*-like invasion phenotype in *P. yoelii* sporozoites. In contrast, complementation of PyΔp52/p36 parasites with PbP52 and PyP36 restored sporozoite infectivity in HepG2/CD81 cells only, but not in HepG2 cells (*Figure 7A and 7B*). This confirms that P52, although essential for sporozoite entry, is not directly associated with host receptor usage. Finally, invasion of PbP36-expressing PyΔp52/p36 sporozoites was abrogated by anti-SR-BI antibodies in HepG2 cells (*Figure 7C*), demonstrating that *P. berghei* P36 is a key determinant of CD81-independent entry via SR-BI.
DISCUSSION

Until now, the nature of the molecular interactions mediating *Plasmodium* sporozoite invasion of hepatocytes has remained elusive. Previous studies have identified CD81 and SR-BI as important host factors for infection of hepatocytes (13,19,20). Still, the relation between CD81 and SR-BI and their contribution to parasite entry was unclear (18,24), and, importantly, parasite factors associated with CD81 or SR-BI usage had not been identified. Here we demonstrate that CD81 and SR-BI define independent entry pathways for sporozoites, and identify the parasite protein P36 as a critical parasite factor that determines host receptor usage during hepatocyte infection.

Our data provide molecular insights into the host entry pathways used by different sporozoite species (*Figure 8A*). We show that *P. falciparum*, like *P. yoelii*, relies on CD81 but not SR-BI, in agreement with the recent observation that antibodies against CD81 but not anti-SR-BI induce protection in humanized mice infected with *P. falciparum* (24). Our results are also consistent with the study from Yalaoui *et al.* showing that in primary mouse hepatocytes antibodies against SR-BI do not inhibit *P. yoelii* infection when co-incubated together with sporozoites (19). In the same study, the authors proposed a model where SR-BI indirectly contributes to *P. yoelii* infection through regulation of membrane cholesterol and CD81 expression, however our data in the HepG2/CD81 cell model with both *P. yoelii* and *P. berghei* clearly rule out a role of SR-BI during CD81-dependent sporozoite entry. For the first time, we also analysed the role of host factors during *P. vivax* sporozoite infection. We found that, in contrary to *P. falciparum*, antibodies against SR-BI but not against CD81 inhibit infection of primary human hepatocyte cultures by *P. vivax* sporozoites, illustrating that the two main species causing malaria in humans use distinct routes to infect hepatocytes.

SR-BI and CD81 both have been shown to bind the HCV envelope glycoprotein E2 (12,21,22), and act in a sequential and cooperative manner to mediate virus entry (37), together with several additional entry factors (38–40). By contrast, as shown here, SR-BI and CD81 operate independently during *Plasmodium* sporozoite infection. Remarkably, *P. berghei* can use alternatively CD81 or SR-BI to infect cells, which is surprising given that
CD81 and SR-BI are structurally unrelated. CD81 belongs to the family of tetraspanins, which are notably characterized by their propensity to dynamically interact with other membrane proteins and organize tetraspanin-enriched membrane microdomains (41). CD81 might play an indirect role during Plasmodium sporozoite entry, possibly by interacting with other host receptors within these microdomains (16,17,42). Interestingly, SR-BI is structurally related to CD36 (43), which is known to bind several Plasmodium proteins, including P. falciparum erythrocyte membrane protein 1 (PfEMP1) in the context of cytoadherence of infected erythrocytes to endothelial cells (44–46). CD36 was also reported to interact with P. falciparum sequestrin (also called LISP2) (47), a member of the 6-cys protein family involved in parasite liver stage development (48,49), and is a major determinant of P. berghei asexual blood stage sequestration (50). CD36 was shown to be dispensable for mouse hepatocyte infection by P. yoelii and P. berghei sporozoites (51). However, it is conceivable that SR-BI, which shares a similar 3D structure as CD36 (43), may interact with parasite proteins expressed by sporozoites, such as the 6-cys protein P36.

The 6-cys protein family is characterized by the presence of a cysteine-rich domain, the 6-cysteine (6-cys) or s48/45 domain. Plasmodium spp. possess a dozen 6-cys proteins, which perform important functions in different life cycle stages, and are often located on the parasite surface, consistent with a role in cellular interactions (49). Previous studies have shown that Plasmodium P52 and P36 are crucial for infection of the liver by sporozoites (6–8), although it remained unclear whether their role was at the sporozoite entry step (7,10) or for maintenance of the PV post-entry (6,33,34). It should be noted that standard invasion assays, as performed in these studies, do not distinguish between sporozoite productive entry and non-productive invasion events associated with cell traversal, complicating the interpretation of phenotypic analysis of the mutants. Here, using GFP-expressing p52 and p36 mutants and a robust FACS-based invasion assay (32), we unequivocally establish that P. yoelii and P. berghei sporozoites lacking P52 and P36 efficiently migrate through cells but do not commit to productive invasion, reproducing the phenotype observed upon blockage of CD81 or SR-BI.
Using a trans-species genetic complementation strategy, we identified P36 as a crucial parasite determinant of host receptor usage. Our data, combined with previous studies (6–9), demonstrate that both P36 and P52 are necessary for sporozoite infection of hepatocytes, irrespective of the invasion route used by the parasite. Our study now reveals that P36 but not P52 is responsible for the phenotypic differences between *P. berghei* and *P. yoelii* sporozoites regarding host cell entry pathways. Importantly, *P. berghei* P36 mediates sporozoite entry via either CD81 or SR-BI, whereas *P. yoelii* P36 only supports CD81-dependent invasion (Figure 8B). These results strongly suggest that PbP36 contains specific structural determinants that confer the ability of the protein to interact with SR-BI or SR-BI-dependent molecules. *P. berghei* and *P. yoelii* P36 proteins share 88% identity and 97% similarity in their amino acid sequence (Figure 8-figure supplement 1). Structural modelling of PbP36, using the crystal structure of the 6-cys protein PfP12 (52) as a template, shows a typical beta sandwich fold for each of the tandem 6-cysteine domains (Figure 8C). Most of the divergent residues between PbP36 and PyP36 are located in the second 6-cys domain (Figure 8-figure supplement 1), including in a loop located between the third and fourth cysteine residues. In this particular loop, PfP36 and PvP36 contain an inserted sequence of 21 and 3 amino acids, respectively, which may affect their binding properties and functions.

Single gene deletions of *p52* or *p36* result in similar phenotypes as *p52/p36* double knockouts, suggesting that the two proteins act in concert (6–8,10). In *P. falciparum* blood stages, the two 6-cys proteins P41 and P12 interact to form stable heterodimers on the surface of merozoites (52–54). Whilst P12 is predicted to be GPI-anchored, P41 lacks a membrane-binding domain, similarly to P36. By analogy, we hypothesize that P36 may form heterodimers with other GPI-anchored 6-cys proteins, including P52. Our data show that complementation of PbΔp52/p36 parasites with P52 and P36 from *P. falciparum* or *P. vivax* does not restore sporozoite infectivity, supporting the idea that other yet unidentified parasite factors cooperate with P52 and P36 during invasion. In addition to P52 and P36, sporozoites express at least three other 6-cys proteins, B9, P12p and P38 (55,56). Whereas gene deletion of *p38* causes no detectable phenotypic defect in *P. berghei* (57), B9 has been
shown to play a critical role during liver stage infection, not only in *P. berghei* but also in *P. yoelii* and *P. falciparum* (49). Whether B9, P38 and P12p associate with P52 and/or P36 and contribute to sporozoite invasion still deserves further investigations.

Several 6-cys proteins have been implicated in molecular interactions with host factors. As mentioned above, sequestrin was reported to interact with CD36 (47), although the functional relevance of this interaction remains to be determined, as sequestrin is only expressed towards the end of liver stage development (48). Recent studies have shown that *P. falciparum* merozoites evade destruction by the human complement through binding of host factor H to the 6-cys protein Pf92 (58,59). Pfs47 expressed by *P. falciparum* ookinetes plays a critical role in immune evasion in the mosquito midgut, by suppressing nitration responses that activate the complement-like system (60,61). Pfs47 was proposed to act as a “key” that allows the parasite to switch off the mosquito immune system by interacting with yet unidentified mosquito receptors (“lock”) (62). By analogy, based on our results, one could envisage P36 as a crucial determinant of a sporozoite “key” that opens SR-BI and/or CD81-dependent “locks” for entry into hepatocytes.

The function of P36 interaction with host cell receptors remains to be defined. P36 binding to SR-BI and/or CD81, either directly or indirectly, may participate in a signalling cascade that triggers rhoptry secretion and assembly of the moving junction, key events committing the parasite to host cell entry (63). Alternatively, P36 may induce signalling in the host cell by acting on SR-BI or other hepatocyte receptors. In this regard, Kaushansky *et al.* recently reported that sporozoites preferentially invade host cells expressing higher levels of the EphA2 receptor (9). Interestingly, this preference was still observed with *p52/p36*-deficient parasites, strongly suggesting that there is no direct link between EphA2 and P52/P36-dependent productive invasion. However, the same study showed that P36 interferes with Ephrin A1-mediated EphA2 phosphorylation (9), raising the possibility that P36 may affect EphA2 signalling indirectly, for example via SR-BI and the SRC pathway (64,65).
In conclusion, our study reveals that host CD81 and SR-BI define two alternative pathways in human cells for sporozoite entry. Most importantly, we identified the parasite 6-cysteine domain protein P36 as a key determinant of host receptor usage during infection. These results pave the way toward the elucidation of the mechanisms of sporozoite invasion. The identification of the parasite ligands that mediate host cell entry may provide potential targets for the development of next-generation malaria vaccines. P36 is required for both CD81- and SR-BI-dependent sporozoite entry, suggesting that it may represent a relevant target in both *P. falciparum* and *P. vivax*. The understanding of host-parasite interactions may also contribute to novel therapeutic approaches. SR-BI-targeting agents have entered clinical development for prevention of HCV graft infection (66). Our data suggest that SR-BI-targeting strategies may be effective to prevent establishment of the liver stages of *P. vivax*, including the dormant hypnozoite forms.
METHODS

Experimental animals, parasites and cells

We used wild type *P. berghei* (ANKA strain, clone 15cy1) and *P. yoelii* (17XLN strain, clone 1.1), and GFP-expressing PyGFP and PbGFP parasite lines, obtained after integration of a GFP expression cassette at the dispensable *p230p* locus (30). *P. berghei* and *P. yoelii* blood stage parasites were propagated in female Swiss mice (6–8 weeks old, from Janvier Labs).

*Anopheles stephensi* mosquitoes were fed on *P. berghei* or *P. yoelii*-infected mice using standard methods (Ramakrishnan et al., 2013), and kept at 24°C and 21°C, respectively. *P. berghei* and *P. yoelii* sporozoites were collected from the salivary glands of infected mosquitoes 21–28 or 14–18 days post-feeding, respectively. *A. stephensi* mosquitoes infected with *P. falciparum* sporozoites (NF54 strain) were obtained from the Department of Medical Microbiology, University Medical Centre, St Radboud, Nijmegen, the Netherlands. *P. vivax* sporozoites were isolated from *A. cracens* mosquitoes, 15-21 days after feeding on blood from infected patients on the Thailand-Myanmar border, as described (67). HepG2 (ATCC HB-8065), HepG2/CD81 (14) and Hepa1-6 cells (ATCC CRL-1830) were checked for the absence of mycoplasma contamination and cultured at 37°C under 5% CO₂ in DMEM supplemented with 10% fetal calf serum and antibiotics (Life Technologies), as described (18). HepG2 and HepG2/CD81 were cultured in culture dishes coated with rat tail collagen I (Becton-Dickinson, Le Pont de Claix, France). Primary human hepatocytes were isolated and cultured as described previously (5).

In vitro infection assays

Primary human hepatocytes (5 x 10⁴ per well in collagen-coated 96-well plates) were infected with *P. vivax* or *P. falciparum* sporozoites (3 x 10⁴ per well), as described (5), and cultured for 5 days before fixation with cold methanol and immunolabeling of EEFs with antibodies specific for *Plasmodium* HSP70 (68). Nuclei were stained with Hoechst 33342 (Life Technologies). Host cell invasion by GFP-expressing *P. berghei* and *P. yoelii* sporozoites was monitored by flow cytometry (69). Briefly, hepatoma cells (3 x 10⁴ per well in
collagen-coated 96-well plates) were incubated with sporozoites (5 x 10^3 to 1 x 10^4 per well).

At different time points, cell cultures were washed, trypsinized and analyzed on a Guava
EasyCyte 6/2L bench cytometer equipped with a 488 nm laser (Millipore), for detection of
GFP-positive cells. To assess liver stage development, HepG2 or HepG2/CD81 cells were
infected with GFP-expressing sporozoites and cultured for 24-36 hours before analysis either
by FACS or by fluorescence microscopy, after fixation with 4% PFA and staining with
antibodies specific for UIS4 (Sicgen) and Hoechst 33342. For antibody-mediated inhibition
assays, we used polyclonal antisera against human SR-BI raised after genetic immunization
of rabbits and rats (70, 71), and monoclonal antibodies against human SR-BI (NK-8H5-E3)
(26), human CD81 (1D6, Abcam) or mouse CD81 (MT81)(16).

**Small interfering RNA and plasmid transfection**

We used small double stranded RNA oligonucleotides targeting human CD81 (5’-
GCACCAAGTGCATCAAGTA-3’), human SR-BI (5’-GGACAAGTTCGGATTATTT-3’ ) or
mouse CD81 (5’-CGTGTCACCTTCAACTGTA-3’). An irrelevant siRNA oligonucleotide
targeting human CD53 (5’-CAACTTCGGAGTGCTCTTC-3’) was used as a control.
Transfection of siRNA oligonucleotides was performed by electroporation, as described (14).
Following siRNA transfection, cells were cultured for 48 hours before flow cytometry analysis
or sporozoite infection. Transfection of pcDNA3 plasmids encoding human CD81 (72) or SR-
BI (70) was performed 24 hours after siRNA using the Lipofectamine 2000 reagent
(Invitrogen) according to the manufacturer’s specifications. Following plasmid transfection,
cells were cultured for an additional 24 hours before sporozoite infection.

**Constructs for targeted gene deletion of p52 and p36**

PbΔp52p36 and PbΔp36 mutant parasites were generated using a “Gene Out Marker Out”
strategy (30). For generation of PbΔp52p36 parasites, a 5’ fragment of PbP52 gene
(PBANKA_1002200) and a 3’ fragment of PbP36 gene (PBANKA_1002100) were amplified
by PCR from P. berghei ANKA WT genomic DNA, using primers PbP52rep1for (5’-
TCCCCGCGGAATCGTGATGCTATGGATAACGTAACAC-3’), PbP52rep2rev (5’-
ATAAGAATGCGGCCGCAAAAAGAGACAAACACACTTTTGTAACACC-3’), PbP36rep3for
(5’-CCGCTCGAGTTAATATGTGATGTTGAGAAGAGATGGAGG-3’) and PbP36rep4rev (5’-
GGGTACCTTGATATGCAACTTTCACATGAGG-3’), and inserted into SacII/NotI and
XhoI/KpnI restriction sites, respectively, of the GOMO-GFP vector1. For generation of
PbΔp36 parasites, a 5’ and a 3’ fragment of PbP36 gene were amplified by PCR from P.
berghei ANKA WT genomic DNA, using primers PbP36repAfor (5’-
AGCTGGAGCTCCACGGGGAAAAAGGTAAACCAATATGGAAAGC-3’), PbP52rep-
Arev (5’-CGGCTGAGGGCGCCGCAATCAAAAAATAATAAAAAACATATGTCACAC-
TCG-3’), and PbP36repBfor (5’-ATTATTTACTCGAGTAGTGTGTGGTGAAGAGT-
GAGG-3’) and PbP36repBrev (5’-TATAGGGCGAATTGGGTACCGCAACCGCAAGAGAATA-
CAATACAAATGG-3’), and inserted into SacII/NotI and Xhol/KpnI restriction sites,
respectively, of the GOMO-GFP vector using the In-Fusion HD Cloning Kit (Clontech).
For generation of PyΔp52p36 parasites, 5’ and 3’ fragments of PyP52 gene
(PY17X_1003600) and a 3’ fragment of PyP36 gene (PY17X_1003500) were amplified by
PCR from P. yoelii 17XNL WT genomic DNA, using primers PyP52rep1for (5’-TCCCCG-
CGGAATCGCCATGCTATGGATAGTGTAGC-3’), PyP52rep2rev (5’-ATAAGAATGCGGCC-
GCCATTGAAGGGAGAAACATCGACGC-3’), PyP52rep3for (5’-CCGCTCGAGTCAAT-
ATGCCACATTCCGTTTGGTGG-3’), PyP52rep4rev (5’-GGGTACCTATTATGGGATAGTCAG-
ATGCACTTTCACATGAGG-3’), PyP36repFor (5’-ATAAGAATGCGCCGCAAAATGCA-
GGGCGTCTTGAACCC-3’) and PyP36repRev (5’-CCGGAATTCCACAAAAAGATGCG-
TACTGAAAGCTTCACC-3’). The fragments were inserted into SacII/NotI, Xhol/KpnI and
NotI/EcoRI restriction sites, respectively, of a GOMO vector backbone containing mCherry
and hDHFR-yFCU cassettes. The resulting targeting constructs were verified by DNA
sequencing (GATC Biotech), and were linearized with SacII and KpnI before transfection.
Wild type P. berghei ANKA blood stage parasites were transfected with pbp52p36 and pbp36
targeting constructs using standard transfection methods (Janse et al., 2006). GFP-
expressing parasite mutants were isolated by flow cytometry after positive and negative
selection rounds, as described(30). PyGFP blood stage parasites were transfected with a
*pyp52pyp36* targeting construct and a GFP-expressing drug selectable marker-free
*PyΔp52p36* mutant line was obtained using a two steps “Gene Out Marker Out” strategy.
Correct construct integration was confirmed by analytical PCR using specific primer
combinations.

**Constructs for genetic complementation of Δp52p36 mutants**

For genetic complementation experiments, we used centromeric plasmids to achieve stable
transgene expression from episomes (36). Complementation plasmids were obtained by
replacing the GFP cassette of pCEN-SPECT2 plasmid (kindly provided by Dr S. Iwanaga)
with a P52/P36 double expression cassette. Four complementing plasmids were generated,
allowing expression of PbP52/PbP36, PyP52/PyP36, PbP52/PyP36 or PyP52/PbP36.
The centromeric plasmid constructs were assembled by In-Fusion cloning of 4 fragments in
two-steps, into KpnI/Sall restriction sites of the pCEN-SPECT2 plasmid. For this purpose,
fragments corresponding to the promoter region of PbP52 (insert A, 1.5 kb), the open
reading frame (ORF) of PbP52 (insert B, 1.7 kb), the 3’ untranslated region (UTR) of PbP52
and promoter region of PbP36 (insert C, 1.5 kb), the ORF and 3’ UTR of PbP36 (insert D, 2
kb), the promoter region of PyP52 (insert E, 1.5 kb), the open reading frame (ORF) of PyP52
(insert F, 1.7 kb), the 3’ untranslated region (UTR) of PyP52 and promoter region of PyP36
(insert G, 1.6 kb), and the ORF and 3’ UTR of PyP36 (insert H, 2 kb) were first amplified by
PCR from *P. berghei* or *P. yoelii* WT genomic DNA, using the following primers: Afor (5’-
TATAGGGCGAATTGGGTACCTTCACATGCATAAACCCGAAGTGTGC-3’), Arev (5’-
GAAAAAAGCAGCTAGCTTGAATGTAGAAAAAATATTATGCGATTTTG-3’), Bfor (5’-
ATTAAAGCAAGCCTAGCATAATATTATATTTATTTATGAGATTTTG-3’), Brev (5’-
GAAAGAGCAGCTAGGTACCTTCACATGCATAAACCCGAAGTGTGC-3’), Cfor (5’-
TGACCTCTCTTTTGGATGTAAGGTAAAAC-3’), Crev (5’-
TTTTTGTACCTCTTTTCTTATTATGATTTTG-3’), Dfor (5’-
GCTAGCTGCTTTTTTCTTTGCGATGGTAGTTAAC-3’), Drev (5’-
TACAAGCATTCTCAATGCAATTATATGCTATAATCTC-3’), Efor (5’-
TATAGGGCGAATTGGGTACCTTCACATGCATAAACCCGAAGTGTGC-3’), Evrev (5’-
GAAAAAAGCAGCTAGCTTGAATGTAGAAAAAATATTATGCGATTTTG-3’), Ffor (5’-
TTTTTGTACCTCTTTTCTTATTATGATTTTG-3’), Frev (5’-
GAAAGAGCAGCTAGGTACCTTCACATGCATAAACCCGAAGTGTGC-3’), Gfor (5’-
TGACCTCTCTTTTGGATGTAAGGTAAAAC-3’), Grev (5’-
TTTTTGTACCTCTTTTCTTATTATGATTTTG-3’), Hfor (5’-
GCTAGCTGCTTTTTTCTTTGCGATGGTAGTTAAC-3’), Hrev (5’-
TACAAGCATTCTCAATGCAATTATATGCTATAATCTC-3’).
Cloning of fragments A and D followed by B and C into the pCEN vector resulted in the PbP52/PbP36 complementation plasmid. Cloning of fragments E and H followed by F and G into the pCEN vector resulted in the PyP52/PyP36 complementation plasmid. Cloning of fragments A and D followed by F and G into the pCEN vector resulted in the PyP52/PbP36 complementation plasmid. Cloning of fragments E and H followed by B and C into the pCEN vector resulted in the PbP52/PyP36 complementation plasmid.

The centromeric plasmids for expression of *P. falciparum* and *P. vivax* P52 and P36 were assembled by In-Fusion cloning of 5 fragments in two steps. For this purpose, fragments corresponding to the promoter region of *PbP52* (insert B1, 1.9 kb), the 3' UTR of *PbP52* and promoter region of *PbP36* (insert B2, 1.6 kb), the 3' UTR of *PbP36* (insert B3, 2 kb), the ORF of *PfP52* (insert F1, 1.4 kb), the ORF of *PfP36* (insert F2, 1.1 kb), the ORF of *PvP52* (insert V1, 1.4 kb) and the ORF of *PvP36* (insert V2, 1.1 kb), were first amplified by PCR from *P. berghei*, *P. falciparum* or *P. vivax* genomic DNA, using the following primers: B1for (5'-TATAGGGCGAATTGGGTACCTTCACATGCATAAACCCGAA-

...
F2rev (5'-ACATCACATAGCCTAGCCTAACTTTCTACAGTTTTATTTATGTTA-AATAAACC-3'), V1for (5'-ATAATAGTAAGCTAGCAAAATGAGGCGGATTCTGCTGGGCTG-3'), V1rev (5'-TGCACATATTGTGATTTACAGGGACGAGAAACCCCGTAG-3'), V2for (5'-TTATTTTTTGTAGTACAGCAGCTCCTTC-CAGTAGTGTGG-3'), and V2rev (5'-ACATCACATAGCTAGCTCACACCGCTTCAACC-GCTGCG-3'). An intermediate vector was first assembled by cloning inserts B1 and B3 into KpnI/SalI restriction sites of the pCEN-SPECT2 plasmid. Subsequently, In-Fusion cloning of inserts F1, B2 and F2 or inserts V1, B2 and V2 into the NheI restriction site of the intermediate vector resulted into PfP52/PfP36 and PvP52/PvP36 expression centromeric plasmids, respectively. All constructs were verified by DNA sequencing (GATC Biotech) before transfection.

**Parasite transfection and selection**

For double crossover replacement of P52 and P36 genes and generation of the PbΔp52p36, PbΔp36 and PyΔp52p36 parasite lines, purified schizonts of wild type *P. berghei* ANKA or PyGFP were transfected with 5-10 μg of linearized construct by electroporation using the AMAXA Nucleofector™ device (program U33), as described elsewhere (73), and immediately injected intravenously in the tail of one mouse. The day after transfection, pyrimethamine (70 or 7 mg/L for *P. berghei* and *P. yoelii*, respectively) was administrated in the mouse drinking water, for selection of transgenic parasites. Pure transgenic parasite populations were isolated by flow cytometry-assisted sorting of GFP and mCherry-expressing blood stage parasites FACSAlia II (Becton-Dickinson), transferred into naïve mice, treated with 1 mg/ml 5-fluorocytosine (Meda Pharma) in the drinking water, and sorted again for selection of GFP+ parasites only, as described (30). In the case of the PyΔp52p36 mutant, GFP+ mCherry+ recombinant parasites were first cloned by injection of limiting dilutions into mice prior to the negative selection step. For genetic complementation of the mutants, purified schizonts of PbΔp52p36 and PyΔp52p36 parasites were transfected with 5 μg of centromeric plasmids, followed by positive selection of transgenic parasites with pyrimethamine, as described above.
Parasite genotyping

Parasite genomic DNA was extracted using the Purelink Genomic DNA Kit (Invitrogen), and analysed by PCR using primer combinations specific for WT and recombinant loci. For genotyping of PbΔp52p36 parasites, we used primer combinations specific for the WT Pbp52 locus (5′-AATGAGATGTCAAAAAAATATAGTGGTCC-3′ and 5′-AAATGAGCAGTTTCTTTACG-TTGTTCC-3′), for the 5′ region of the recombinant locus (5′-TATGTTTGGAATATCAGG-ACAAGGCATGG-3′ and 5′-TAATAATTTGAGTCTTTTAGTAACAGATGGCC-3′), and for the 3′ region of the recombinant locus before (5′-ATCGTGGAACAGTACGAACGGCGCAGG-3′ and 5′-ATTGGACGTATTATTAGGAAAGCC-3′) or after excision of the selectable marker (5′-GATGGGAACGCTTCACAGCAGACC-3′ and 5′-ATTGGACGTATTATTAGCAGGCC-3′). For genotyping of PbΔp36 parasites, we used primer combinations specific for the WT Pbp36 locus (5′-GAGTTGCAACGCATTTAGTG-3′ and 5′-CCATGATGAGATGCTAAATCGGG-3′), for the 5′ region of the recombinant locus (5′-GGAAGCATCATACAAAAAGAAAAGC-3′ and 5′-TAATAATTTGAGTCTTTAGTAACGACCA-3′), and for the 3′ region of the recombinant locus before (5′-ATCGTGGAACAGTACGAACGGCGCAGG-3′ and 5′-CGTTATCTCTTTTTTTTACTCATTAGATGTTG-3′) or after excision of the selectable marker (5′-GATGGGAACGCTTCACAGCAGACC-3′ and 5′-CGTTATCTCTTTTTTTTACTCATTAGATTTG-3′). For genotyping of PyΔp52p36 parasites, we used primer combinations specific for the WT PyP52 locus (5′-ACTATATTTCAATTGGAGACATGTGG-3′ and 5′-ATGCAAAAAAGTTATCATTGCTAGTTGG-3′), for the 5′ region of the recombinant locus (5′-GTATGTTTGGAATGCCAGGATATGACATGG-3′ and 5′-CCGGAATTCACAAAAAGATCAGTTGG-3′), for the 5′ region of the recombinant locus before (5′-AGTTACACGTATATTACGACCATGAGATG-3′ and 5′-TAAGCATATATTGTATTAGGCTTTGAC-3′) or after excision of the selectable marker (5′-
GTATGTTTGAATGCCAGGATATGACATGG-3' and 5'-AATCTGATATGATAAATTATG-GTATTGGAC-3').

Bioinformatic and structural analysis

Amino-acid sequences of the P36 proteins from *P. falciparum* (379 aa, gi:296004390, PF3D7_0404400), *P. vivax* (320 aa, gi:156094683, PVX_001025), *P. berghei* (352 aa, gi:991456178, PBANKA_1002100) and *P. yoelii* (356 aa, gi:675237743, PY17X_1003500) were obtained from Genbank. Sequence alignments were carried out using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/). A 3D model of *P. berghei* P36 was generated by the prediction program I-Tasser (74), using the 3D structure of Pf12 (Pf12short in ref (52)), which contains two 6-cys domains D1 and D2 arranged in tandem, as a template (PDB access code 2YMO). The 3D model for PbP36 was then superimposed to the template Pf12short and visually inspected using the program Coot (75), and the rotamers for the Cys residues adjusted such that the three disulfides bonds for each domain were formed following the pattern C1-C2, C3-C6 and C4-C5.

Statistical analysis

Statistical significance was assessed by non-parametric analysis using the Mann-Whitney U and Kruskal-Wallis tests. All statistical tests were computed with GraphPad Prism 5 (GraphPad Software). Significance was defined as p<0.05 (ns, statistically non-significant; *p<0.05; **p<0.01). In vitro experiments were performed at least three times, with a minimum of three technical replicates per experiment.

Ethics statement

All animal work was conducted in strict accordance with the Directive 2010/63/EU of the European Parliament and Council ‘On the protection of animals used for scientific purposes’. The protocol was approved by the Charles Darwin Ethics Committee of the University Pierre et Marie Curie, Paris, France (permit number Ce5/2012/001). Blood samples were obtained
from *P. vivax*-infected individuals attending the Shoklo Malaria Research Unit (SMRU) clinics on the western Thailand-Myanmar border, after signature of a consent form(67). Primary human hepatocytes were isolated from healthy parts of human liver fragments, which were collected from adult patients undergoing partial hepatectomy (Service de Chirurgie Digestive, Hépato-Bilio-Pancréatique et Transplantation Hépatique, Groupe Hospitalier Pitié-Salpêtrière, Paris, France). The collection and use of this material were undertaken in accordance with French national ethical guidelines under Article L. 1121-1 of the ‘Code de la Santé Publique’, and approved by the Institutional Review Board (Comité de Protection des Personnes) of the Centre Hospitalo-Universitaire Pitié-Salpêtrière, Assistance Publique-Hôpitaux de Paris, France.

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COMPETING FINANCIAL INTERESTS STATEMENT

The authors declare no competing financial interests.

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FIGURE LEGENDS

Figure 1. Anti-SR-BI antibodies inhibit *P. vivax* but not *P. falciparum* sporozoite infection. **A.** Primary human hepatocyte cultures were incubated with *P. vivax* sporozoites in the presence of anti-CD81 and/or anti-SR-BI mAbs at 20 μg/ml, and the number of EEF-infected cells was determined 5 days post-infection after labeling of the parasites with anti-HSP70 antibodies. **B.** Primary human hepatocytes were incubated with *P. vivax* sporozoites in the presence or absence of anti-SR-BI polyclonal rabbit serum (diluted 1/100), and the number of EEFs was determined at day 5 as by immunofluorescence. **C.** Primary human hepatocyte cultures were incubated with *P. falciparum* sporozoites in the presence of anti-CD81 mAb (20 μg/ml) and/or anti-SR-BI polyclonal rabbit serum (diluted 1/100), and the number of EEF-infected cells was determined 5 days post-infection after labeling of the parasites with anti-HSP70 antibodies. Results from 3 independent experiments are shown and expressed as the percentage of control (mean +/- SD). **D.** Immunofluorescence analysis of *P. vivax* EEFs at day 5 post-infection of primary human hepatocytes. Parasites were labeled with anti-HSP70 antibodies (green), and nuclei were stained with Hoechst 33342 (blue). Large EEFs and small EEFs were observed in both control and anti-SR-BI antibody-treated cultures. Scale bars, 10 μm. **E.** Inhibitory activity of anti-SR-BI antibodies on small EEFs (white histograms) and large EEFs (black histograms). The results from 4 independent experiments are shown, and are expressed as the percentage of inhibition observed with anti-SR-BI antibodies as compared to the control.

Figure 2. Infection of human HepG2 cells by *P. berghei* sporozoites depends on SR-BI. **A.** HepG2 cells were incubated with *P. berghei* sporozoites for 3 h in the absence (Control) or presence of increasing concentrations of rabbit polyclonal SR-BI antisera. Infected cultures were further incubated for 24h before quantification of EEFs-infected cells by fluorescence microscopy. **B.** HepG2 cell cultures were infected with PbGFP sporozoites as in A, and the number of invaded cells (GFP+) was quantified by FACS 3 hours post-infection.
**Figure 3. CD81 and SR-BI define alternative entry routes for *P. berghei* sporozoites.**

A. HepG2/CD81 cells were incubated with *P. berghei* sporozoites in the presence or absence of anti-human CD81 and/or SR-BI mAbs, and the number of EEFs-infected cells was determined by fluorescence microscopy 24 hours post-infection. B. *P. berghei* EEF numbers in HepG2/CD81 cells transfected with siRNA oligonucleotides targeting CD81 (siCD81) and/or SR-BI (siSR-BI). C. HepG2/CD81 cells were incubated with *PbGFP* sporozoites for 15-120 min, in the presence or absence of anti-CD81 and/or anti-SR-BI antibodies, then trypsinized and directly analyzed by FACS to quantify invaded (GFP-positive) cells. D. Protein extracts from Hepa1-6 cells and Hepa1-6 cells transiently transfected with a human SR-BI expression plasmid were analyzed by Western blot using antibodies recognizing mouse and human SR-BI (Abcam) or mouse CD81 (MT81). E. Hepa1-6 cells were transfected first with siRNA oligonucleotides targeting endogenous mouse CD81 (simCD81), then with plasmids encoding human CD81 (hCD81) or SR-BI (hSR-BI). Cells were then incubated with *PbGFP* sporozoites, and the number of infected (GFP-positive) cells was determined 24h post-infection by FACS.
Figure 4. Infection of HepG2/CD81 cells by *P. yoelii* sporozoites depends on CD81 but not SR-BI. A. HepG2/CD81 cells were incubated with *P. yoelii* sporozoites in the presence of anti-CD81 mAb (20 μg/ml) or anti-SR-BI polyclonal rabbit serum (diluted 1/100), and the number of EEF-infected cells was determined 24 hours post-infection by fluorescence microscopy. Results from 3 independent experiments are shown and expressed as the percentage of control (mean +/- SD). B. *P. yoelii* EEF numbers in HepG2/CD81 cells transfected with siRNA oligonucleotides targeting CD81 (siCD81) and/or SR-BI (siSR-BI).

Figure 5. The 6-cys proteins P52 and P36 are required for productive host cell invasion. A-B. HepG2 (A) or Hepa1-6 cells (B) were incubated with PbGFP (blue lines) or PbΔp52/p36 sporozoites (red lines) for 15-120 minutes, in the presence (dotted lines) or absence (solid lines) of anti-SR-BI (A) or anti-CD81 (B) antibodies. Cells were then trypsinized and directly analyzed by FACS to quantify invaded (GFP-positive) cells. C. HepG2 and HepG2/CD81 cells were infected with PbGFP or PbΔp52/p36 sporozoites and the number of EEFs was determined 28 hours post-infection by fluorescence microscopy. D. HepG2 and HepG2/CD81 cells infected with PbGFP or PbΔp52/p36 sporozoites were fixed at 28 hours post-infection, stained with anti-UIS4 antibodies (red) and the nuclear stain Hoechst 33342 (blue), and examined by fluorescence microscopy. Parasites were detected based on GFP expression (green). Scale bars, 10 μm. E. Quantification of UIS4 expression in HepG2 and HepG2/CD81 cells infected with PbGFP (red) or PbΔp52/p36 (black). F. HepG2 (dotted lines) and HepG2/CD81 cells (solid lines) were incubated with PyGFP (blue lines) or PyΔp52/p36 sporozoites (red lines) for 15-180 minutes, trypsinized, and directly analyzed by FACS to quantify invaded (GFP-positive) cells. G. HepG2/CD81 cells were infected with PyGFP or PyΔp52/p36 sporozoites and the number of EEFs was determined 24 hours post-infection by fluorescence microscopy. H. HepG2 and HepG2/CD81 cells infected with PyGFP or PyΔp52/p36 sporozoites were fixed at 24 hours post-infection, stained with anti-UIS4 antibodies (red) and the nuclear stain Hoechst 33342 (blue), and examined by fluorescence microscopy. Parasites were detected based on GFP expression (green). Scale
bars, 10 µm. I. Quantification of UIS4 expression in HepG2 and HepG2/CD81 cells infected with PyGFP (red) or PyΔp52/p36 (black).

**Figure 6.** P36 mediates CD81-independent entry in *P. berghei* sporozoites. A. HepG2 (blue histograms) or HepG2/CD81 cells (red histograms) were incubated with sporozoites from PbΔp52/p36 parasites genetically complemented with *P. berghei* and/or *P. yoelii* P52 and P36, and analysed by FACS or fluorescence microscopy to determine the number of GFP-positive cells 24h post-infection. Results from 3 independent experiments are shown and are expressed as the percentage of infection in comparison to control PbGFP-infected cultures (mean +/-SD). B. Immunofluorescence analysis of UIS4 expression in HepG2 or HepG2/CD81 cells infected with genetically complemented PbΔp52/p36 sporozoites. Cells were fixed with PFA 28 hours post-infection, permeabilized, and stained with anti-UIS4 antibodies (red) and the nuclear stain Hoechst 33342 (blue). Parasites were detected based on GFP expression (green). Scale bars, 10 µm. C-D. HepG2/CD81 (C) and HepG2 (D) cells were infected with PbGFP, PbΔp52/p36 and complemented PbΔp52/p36 sporozoites. The numbers of UIS4-positive (red histograms) and UIS4-negative (black histograms) EEFs were determined by fluorescence microscopy 24 hours post-infection.

**Figure 7.** Transgenic *P. yoelii* sporozoites expressing *PbP36* can infect CD81-null cells via SR-B1 A. HepG2 (blue) and HepG2/CD81 cells (red) were incubated with genetically complemented PyΔp52/p36 sporozoites, and fixed 24h post-infection. The number of UIS4-positive vacuoles was then determined by immunofluorescence. B. Immunofluorescence analysis of UIS4 expression in HepG2 or HepG2/CD81 cells infected with sporozoites of PyΔp52/p36 parasites genetically complemented with P52 and P36 from *P. berghei* or *P. yoelii*. Cells were fixed with PFA, permeabilized, and stained with anti-UIS4 antibodies (red) and the nuclear stain Hoechst 33342 (blue). Parasites were detected based on GFP expression (green). Scale bars, 10 µm. C. Quantification of UIS4 expression in HepG2 (blue) and HepG2/CD81 cells (red) infected with genetically complemented PyΔp52/p36
sporozoites and processed as in B for immunofluorescence. D. HepG2 cells were incubated with PyΔp52/p36 sporozoites complemented with PbP36 and either PbP52 or PyP52, in the presence or absence of anti-SR-BI antibodies. Infected cultures were fixed 24h post-infection, and the number of EEFs was then determined by fluorescence microscopy.

**Figure 8. Model of host cell entry pathways for *Plasmodium* sporozoites.** A. Host cell membrane proteins CD81 and SR-BI define two independent entry routes for *Plasmodium* sporozoites. *P. falciparum* and *P. yoelii* sporozoites require CD81 for infection, whereas *P. vivax* sporozoites infect hepatocytes using SR-BI. *P. berghei* sporozoites can enter cells alternatively via CD81 or SR-BI. B. The 6-cysteine domain protein P36 determines host cell receptor usage during *P. yoelii* and *P. berghei* sporozoite invasion. Whilst PyP36 supports only CD81-dependent sporozoite entry, PbP36 mediates sporozoite invasion through both CD81- and SR-BI-dependent pathways. C. Model of the 3D structure of *P. berghei* P36, established based on the crystal structure of PfP12 (2YMO). In the ribbon diagram, the tandem 6-cysteine domains are shown in blue (D1) and green (D2), respectively, and the cysteine residues and disulphide bonds in yellow. The loop located between the third and fourth cysteine residues of the D2 domain (inter-cys loop) is indicated in red.
SUPPLEMENTAL FIGURE LEGENDS

Figure 2-figure supplement 1. CD81 and SR-BI surface expression in HepG2 and HepG2/CD81 cells. HepG2 (A) and HepG2/CD81 cells (B) were stained with anti-CD81 (red histograms) or anti-SR-BI (green histograms) antibodies and analyzed by FACS.

Figure 2-figure supplement 2. Anti-SR-BI antibodies neutralize *P. berghei* infection of HepG2 cells. Effect of anti-SR-BI rat polyclonal antibodies (pAb) and mouse mAb on *P. berghei* EEF numbers in HepG2 cells.

Figure 2-figure supplement 3. Effect of SR-BI silencing on sporozoite cell traversal and invasion. HepG2 cells transfected with siRNA oligonucleotides targeting SR-BI (siSR-BI) or a control siRNA (siCont) were incubated for 3 hours with PbGFP sporozoites and rhodamine-labeled dextran, and the percentage of invaded cells (GFP-positive, green bars) and traversed cells (dextran-positive, red bars) was determined by FACS.

Figure 3-figure supplement 1. Effect of anti-CD81 and anti-SR-BI antibodies on *P. berghei* sporozoite cell traversal and intracellular development. A. HepG2/CD81 cells were incubated for 3 hours with PbGFP sporozoites and rhodamine-labeled dextran, in the presence or absence of anti-CD81 and/or SR-BI antibodies. The percentage of traversed (dextran-positive) cells was then determined by FACS. B. HepG2/CD81 cells were infected with PbGFP sporozoites in the presence or absence of anti-CD81 or anti-SR-BI antibodies, and incubated for 24 hours before measurement of the MFI of infected (GFP-positive) cells by FACS.

Figure 5-figure supplement 1. Targeted gene deletion of p52 and p36 in *P. berghei*. A. Replacement strategy to generate PbΔp52p36 parasites. The wild-type (WT) genomic locus of *P. berghei* p52/p36 was targeted with a GOMO-GFP replacement plasmid containing a 5'
and a 3' homologous sequence inserted on each side of the plasmid GFP/hDHFR-yFCU/mCherry triple cassette. Upon double crossover recombination, the adjacent p52 and p36 genes are replaced by the plasmid cassettes. Subsequent recombination between the two identical PbDHFR/TS 3' UTR sequences (pink lollipops) results in excision of hDHFR-yFCU and mCherry. Genotyping primers and expected PCR fragments are indicated by arrows and lines, respectively. **B-C.** PCR analysis of genomic DNA isolated from control PbGFP and PbΔp52p36 parasites recovered after positive selection with pyrimethamine (B) and after negative selection with 5-fluorocytosine (C). Confirmation of the predicted recombination events was achieved with primer combinations specific for 5' integration (5' integr.), 3' integration (3' integr.) or 3' integration followed by marker excision (3' excised). Primers used for genotyping are indicated in the Materials and Methods. The absence of amplification with primer combinations specific for the WT locus (WT) and the non-excised integrated construct (3' integration) confirms that the final populations contain only PbΔp52p36 drug-selectable marker-free *P. berghei* parasites.

**Figure 5-figure supplement 2. Targeted gene deletion of p52 and p36 in *P. yoelii*.**

Replacement strategy to generate PyΔp52p36 parasites. The wild-type (WT) genomic locus of *P. yoelii* p52/p36 in the PyGFP parental line was targeted with a GOMO replacement plasmid containing a 5' and a 3' homologous sequence from *pyp52* inserted on each side of a hDHFR-yFCU/mCherry double cassette. An additional 3' homologous sequence from *pyp36* (36-3') was inserted immediately downstream of the 5' homologous sequence from *pyp52* (52-5'). Upon double crossover recombination, *pyp52* is replaced by the plasmid cassettes. Subsequent recombination between the two identical 36-3' sequences results in excision of hDHFR-yFCU, mCherry and *pyp36*. Genotyping primers and expected PCR fragments are indicated by arrows and lines, respectively. **B-C.** PCR analysis of genomic DNA isolated from parental PyGFP and PyΔp52p36 parasites recovered after positive selection with pyrimethamine (B) and after negative selection with 5-fluorocytosine (C). Confirmation of the predicted recombination events was achieved with primer combinations.
specific for 5' integration (5' integr.), 3' integration (3' integr.) or 3' integration followed by marker excision (3' excised). Primers used for genotyping are indicated in Materials and methods. The absence of amplification with primer combinations specific for the WT locus (WT) and the non-excised integrated construct (3' integration) confirms that the final populations contain only PyΔp52p36 drug-selectable marker-free *P. yoelii* parasites.

**Figure 5-figure supplement 3. Targeted gene deletion of p36 in *P. berghei*.**

A. Replacement strategy to generate PbΔp36 parasites. The wild-type (WT) genomic locus of *P. berghei p36* was targeted with a GOMO-GFP replacement plasmid containing a 5' and a 3' homologous sequence inserted on each side of the plasmid GFP/hDHFR-yFCU/mCherry triple cassette. Upon double crossover recombination, the p36 gene is replaced by the plasmid cassettes. Subsequent recombination between the two identical PbDHFR/TS 3' UTR sequences (pink lollipops) results in excision of hDHFR-yFCU and mCherry. Genotyping primers and expected PCR fragments are indicated by arrows and lines, respectively. B-C. PCR analysis of genomic DNA isolated from control PbGFP and PbΔp36 parasites recovered after positive selection with pyrimethamine (B) and after negative selection with 5-fluorocytosine (C). Confirmation of the predicted recombination events was achieved with primer combinations specific for 5' integration (5' integr.), 3' integration (3' integr.) or 3' integration followed by marker excision (3' excised). Primers used for genotyping are indicated in the Materials and methods. The absence of amplification with primer combinations specific for the WT locus (WT) and the non-excised integrated construct (3' integration) confirms that the final populations contain only PbΔp36 drug-selectable marker-free *P. berghei* parasites.

**Figure 5-figure supplement 4. P36 is required for *P. berghei* sporozoite entry via both SR-BI- and CD81-dependent routes.** HepG2 and HepG2/CD81 cells were incubated with PbGFP or PbΔp36 sporozoites in the presence or absence of anti-CD81 and anti-SR-BI
antibodies, and the percentage of infected (GFP-positive) cells was determined 24 hours post-infection by FACS.

**Figure 6-figure supplement 1. Genetic complementation with p52 and p36 from P. falciparum or P. vivax does not restore sporozoite infectivity in PbΔp52p36 parasites.**

A. HepG2 and HepG2/CD81 cells were incubated with PbGFP, PbΔp52p36 and PbΔp52p36 complemented with p52 and p36 from P. falciparum or P. vivax. The percentage of infected (GFP-positive) cells 24 hours post-infection was determined by FACS. B. Primary human hepatocytes were incubated with PbGFP, PbΔp52p36 and PbΔp52p36 complemented with p52 and p36 from P. falciparum or P. vivax. The number of EEFs was determined 24 hours post-infection by fluorescence microscopy.

**Figure 8-figure supplement 1. P36 protein sequence analysis.** A. Alignment of P. berghei and P. yoelii P36 protein sequences. Identical, similar and different amino acids are shaded in black, grey and red, respectively. The tandem 6-cys domains D1 and D2 are indicated with blue and green lines, respectively, above the protein sequences. The six cysteine residues of each domain are indicated below the protein sequences. B. Schematic representation of the tandem D1 and D2 6-cys domains of P36, showing the disulfide bond arrangement. The position of the "inter-cys loop", located between the third and fourth cysteine residues of D2, is indicated as a red line. C. Alignment of P. falciparum, P. vivax, P. berghei and P. yoelii inter-cys loop sequences. Identical and similar amino acids are shaded in black and grey, respectively.
A

Infection rate (% of PbGFP)

PbΔp52p36 complementation

HepG2  |  HepG2/CD81

Pb52  |  PbP52 + Pb36
Py52  |  PyP52 + Py36
Pb52  |  PbP52 + Pb36
Py52  |  PyP52 + Py36

B

UIS4-negative  |  UIS4-positive

PbGFP  |  PbP52  |  PbP52 + Pb36
PbP52  |  PyP52  |  PyP52 + Py36

PbP52 + Pb36 complemented

D

UIS4-negative  |  UIS4-positive

PbGFP  |  PbP52  |  PbP52 + Pb36
PbP52  |  PyP52  |  PyP52 + Py36

PbP52 + Pb36 complemented
**A**

![Bar chart showing P. yoelii UIS4+ EEFs for HepG2 and HepG2/CD81 cells with different complementation conditions.](chart_A)

**B**

![Immunofluorescence images of HepG2 and HepG2/CD81 cells with PbP52 and PyP36 complementation.](chart_B)

**C**

![Bar chart showing % UIS4-positive parasites for HepG2 and HepG2/CD81 cells with different complementation conditions.](chart_C)

**D**

![Bar chart showing P. yoelii EEFs for control and Anti-SR-BI conditions with different complementation conditions.](chart_D)
A

% GFP-positive cells

PbGFP  
Ø  
+  
PfP52  
PvP52  
PfP36  
PvP36  
PbΔp52p36 complementation

B

EEFs

PbGFP  
Ø  
+  
PfP52  
PvP52  
PfP36  
PvP36  
PbΔp52p36 complementation