

# GLUT1-mediated glucose uptake plays a crucial role during *Plasmodium* hepatic infection

**Running Title:** Glucose uptake by GLUT1 during *Plasmodium* liver infection

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## Summary

Intracellular pathogens have evolved mechanisms to ensure their survival and development inside their host cells. Here, we show that glucose is a pivotal modulator of hepatic infection by the rodent malaria parasite *Plasmodium berghei* and that glucose uptake via the GLUT1 transporter is specifically enhanced in *P. berghei*-infected cells. We further show that ATP levels of cells containing developing parasites are decreased, which is known to enhance membrane GLUT1 activity. In addition, GLUT1 molecules are translocated to the membrane

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of the hepatic cell, increasing glucose uptake at later stages of infection. Chemical inhibition of GLUT1 activity leads to a decrease in glucose uptake and the consequent impairment of hepatic infection, both *in vitro* and *in vivo*. Our results reveal that changes in GLUT1 conformation and cellular localization seem to be part of an adaptive host response to maintain adequate cellular nutrition and energy levels, ensuring host cell survival and supporting *P. berghei* hepatic development.

## Introduction

Glucose is the primary source of energy and a key substrate for most cells. Glucose and other carbohydrates are transported into cells by members of a family of integral membrane glucose transporter (GLUT) molecules. To date 14 members of this family, also called the solute carrier 2A (SLC2A) proteins, have been identified in humans, which are divided on the basis of transport characteristics and sequence similarities into several families (Classes I to III) [reviewed in (Karim et al., 2012; Mueckler and Thorens, 2013)]. GLUT1 is a class I facilitative glucose transporter expressed in liver cells (Tal et al., 1990) and overexpressed in various tumors (Smith, 1999). GLUT1 expression is highest in the human erythrocyte membrane, and has been shown to play a critical role in cerebral glucose uptake (Koranyi et al., 1991). GLUT1 is also a receptor for the human T cell leukemia virus (HTLV) (Manel et al., 2003) and GLUT1-mediated glucose transport in T-cells has been shown to regulate HIV infection (Loisel-Meyer et al., 2012).

Mammalian infection by the malaria parasite is initiated when *Plasmodium* sporozoites, injected through the bite of an infected mosquito, cross the endothelium of the liver sinusoids and enter the liver. Sporozoites then traverse a few hepatocytes before productively invading a final one, inside which they asymptotically differentiate into exoerythrocytic forms (EEFs) that originate thousands of red blood cell-infective merozoites (Prudencio et al., 2006). Merozoites are eventually released to the blood stream, initiating the blood stage of

infection, and giving rise to malaria symptoms. The liver stage of a mammalian infection by *Plasmodium* is an obligatory uni-directional step in the parasite's progression towards the symptomatic, erythrocytic phase of its life cycle.

Blood stages of malarial parasites are dependent on glycolysis, employed as their main energy source of ATP production (Kirk et al., 1996; Pfaller et al., 1982; Vander Jagt et al., 1990), a process that has recently been modeled for the human malaria parasite *P. falciparum* (Penkler et al., 2015). Glucose has been shown to be transported from human blood plasma into the erythrocyte cytosol by GLUT1 (Hellwig and Joost, 1991), and then taken up by the parasite via a parasite-encoded facilitative hexose transporter (PfHT) (Joet et al., 2003; Woodrow et al., 2000), which limits the rate of glucose entry into the parasite's glycolytic pathways (Tjhin et al., 2013). These findings support novel chemotherapeutic interventions that target PfHT (Feistel et al., 2008; Slavic et al., 2011b). Studies in the rodent malaria model, *P. berghei*, further found that the orthologous hexose transporter (PbHT) is expressed not only throughout the parasite's development within the mosquito vector, but also during liver and transmission stages of *P. berghei* (Slavic et al., 2011a; Slavic et al., 2010). During their extensive hepatic replication, developing *Plasmodium* parasites require the availability of large amounts of nutrients and energy. The availability of appropriate amounts of glucose in infected hepatic cells is thus expected to play an essential role in the development of liver stage *Plasmodium* parasites. Inhibition of PbHT by compound 3361, a specific inhibitor of plasmodial HTs, impairs hepatic *P. berghei* development (Slavic et al., 2011a), supporting the essentiality of glucose uptake for *Plasmodium* liver stages. Accordingly, a recent study established glucose concentration in the culture medium as a key factor for liver stage parasite development (Itani et al., 2014).

In this study, we employed rodent *P. berghei* parasites, a well-established model of malaria infection (Prudencio et al., 2011), to elucidate the uptake and utilization of glucose by *Plasmodium* liver stages. We investigated the glucose requirements during the course of *P. berghei* development in hepatic cells as well as the host molecular receptors involved in

glucose uptake by those cells. We demonstrate that *P. berghei* infection leads to intracellular ATP depletion and enhances the translocation of GLUT1 to the cell membrane of infected hepatoma cells, allowing the uptake of significantly higher amounts of glucose compared with non-infected cells. We further show that glucose plays an essential role during the liver stage of infection by the malaria parasite, modulating its liver stage development both *in vitro* and *in vivo*.

## Results

### Effect of glucose on *P. berghei* hepatic infection

In order to evaluate glucose requirements throughout the liver stage of infection, Huh7 cells, a human hepatoma cell line, were infected with luciferase-expressing *P. berghei* sporozoites in the presence of varying concentrations of glucose. Glucose concentrations ranged from 1.25 to 20 mM, which includes and expands the physiological range of glucose concentrations, 2.5 to 10 mM (Shrayyef, 2010). Luminescence intensity, a correlate of parasite load (Ploemen et al., 2009), and cell viability were measured near the end of hepatic parasite development, at 48 hours post-infection (hpi). Our results show that an increase in glucose availability correlates with an increase of overall *Plasmodium* infection (Fig. 1A). In contrast, any glucose concentration below the standard medium  $\approx 10$  mM concentration significantly impairs infection (Fig. 1A), demonstrating that glucose is required for a successful hepatic *Plasmodium* infection. Cell viability was not affected by the presence of an excess of glucose in the medium, but decreased for 2.5 and 1.25 mM of glucose (Fig. 1A). Crucially, decreasing the glucose concentration from 10 to 5 mM significantly decreases parasite load but has no impact on cell viability.

In order to dissect how glucose influences hepatic infection, we employed an established flow cytometry-based approach that makes use of green fluorescent protein (GFP)-expressing *P. berghei* parasites to determine the number of infected cells and to measure

parasite development (Prudencio et al., 2008). The analysis of cells 2 h after sporozoite addition, when the invasion process is virtually completed (Prudencio et al., 2008), shows that glucose concentration does not affect the ability of parasites to traverse (data not shown) or invade (Fig. 1B) cells. Conversely, when cells were analyzed 48 hpi, a glucose-dependent increase in parasite development was observed (Fig. 1C). These results also showed that the number of infected cells is higher at 20 mM glucose concentration, and lower at glucose concentrations below the physiological range, suggesting that glucose availability influences the survival of infected cells. These results were further confirmed by immunofluorescence microscopy analysis, which demonstrated that parasite size is proportional to the amount of glucose in the medium and that the survival of liver stage parasites 48 hpi depends on the availability of glucose (Fig. 1D, E). In fact, at low glucose concentrations, not only are most parasites very small ( $<50 \mu\text{m}^2$ ) but also the number of infected cells is very low. Increasing concentrations of glucose lead to higher numbers of parasites and favor parasite growth, with approximately 40% of the parasites being larger than  $200 \mu\text{m}^2$  at 10 and 20 mM glucose (Fig. 1E).

Whereas hepatoma cells are highly dependent on glucose uptake for ATP production through glycolysis (Kroemer and Pouyssegur, 2008; Warburg et al., 1927), primary hepatocytes are able to store glucose as glycogen and use oxidative phosphorylation to produce ATP. Accordingly, the requirements for glucose uptake by primary hepatic cells are significantly lower than those displayed by tumorigenic cells (O'Neil et al., 2005). In agreement with this, we showed that the decreased cell viability noted in hepatoma cells at 2.5 and 1.25 mM glucose was not observed in primary hepatocytes, even upon complete removal of glucose. Crucially, however, our data also clearly show that overall infection of mouse primary hepatocytes by *P. berghei* is significantly decreased in the absence of glucose in the medium (Fig. S1). Overall, these results demonstrate that glucose availability plays a crucial role in the liver stage development and survival of *Plasmodium* parasites.

## **Glucose uptake is specifically increased in hepatic cells containing replicating *P.***

### ***berghei* parasites**

Having demonstrated a requirement for glucose during liver stage *P. berghei* development, we then sought to determine whether glucose uptake is specifically enhanced in infected cells and whether this is linked to parasite replication within these cells. To this end, we monitored by flow cytometry the uptake of a fluorescent glucose derivative, 2-NBDG (2-deoxy-2-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]-D-glucose) (O'Neil et al., 2005; Yamada et al., 2007) by *P. berghei*-infected cells. Huh7 cells were incubated with 2-NBDG and analyzed at different time points following addition of red fluorescence protein (RFP)-expressing *P. berghei* sporozoites. The uptake of 2-NBDG was measured as an increase in green fluorescence intensity, whereas parasite development was monitored as a function of red fluorescence intensity. The latter enables the population of cells containing replicating parasites to be distinguished from a smaller population of cells containing parasites that do not develop *in vitro* (Prudencio et al., 2008) (Fig. S2). Our results clearly show that glucose uptake is highly increased in cells containing developing parasites, from 30 hpi onwards (Fig. 2A). A smaller increase in glucose uptake is also observed at around the same time in non-infected cells and in cells containing non-developing parasites, present in the same well (Fig. 2A). Live fluorescence microscopy analysis of non-infected and infected Huh7 cells 48 hpi (Fig. S3A) confirmed that 2-NBDG fluorescence intensity is significantly higher in infected than in non-infected cells (Fig. S3B) and further revealed the uptake of 2-NBDG by the developing parasite (Fig. S3B).

Since it is known that glucose uptake may be influenced by factors such as feeding/fasting, exposure to low/high temperature, exercise, oxidative stress, several liver pathologies, such as steatosis and non-alcoholic fatty liver disease (NAFLD), and liver infections, for example, by hepatitis C virus (HCV) (Bechmann et al., 2012; Bitar et al., 2005; Cunningham et al., 1985; Moore et al., 2012; Pencek et al., 2004; Vidyashankar et al., 2012; Yu et al., 2013), we sought to assess the specificity of the *P. berghei*-induced increase in glucose

uptake by infected Huh7 cells. To this end, we measured 2-NBDG uptake by Huh7 cells subjected to conditions known to induce low-temperature stress in various types of mammalian cells (Fujita, 1999), high-temperature stress in a chicken hepatocellular carcinoma cell line (Sun et al., 2015) or oxidative stress in rat hepatocytes (Nourissat et al., 2008; Sergent et al., 2005) (Fig. 2B). Finally, we infected Huh7 cells with murine gammaherpesvirus 68 (MHV-68) (Collins et al., 2009), and measured 2-NBDG uptake at 24 hpi and at different infection loads (Fig. 2C). The efficacy of infection was confirmed by flow cytometry employing YFP-expressing MHV-68 in a parallel experiment (Fig. S4). Our data clearly show that there was little or no effect on 2-NBDG uptake into the cells by any of the stress-inducing treatments employed, or by viral infection. This indicates that the increase in glucose uptake by *P. berghei*-infected cells is not the result of a non-specific response to stress or to infection (Fig. 2B, C). Overall, our results show that *P. berghei* development inside Huh7 leads to a specific and marked enhancement of glucose uptake by these cells. The observed effect appears rather unique to malaria parasites, as other intracellular pathogens, such as *Toxoplasma gondii* do not depend on host-derived glucose (Blume et al., 2009), and HCV replication actually suppresses cellular glucose uptake through down-regulation of cell surface expression of glucose transporters (Kasai et al., 2009).

### **RNAi screen implicates GLUT1 in glucose uptake by *P. berghei*-infected hepatic cells**

The results so far suggest that infection of hepatic cells by *Plasmodium* modulates the uptake of glucose by these cells, possibly through the enhancement of the activity of membrane glucose transporters in the cell. To ascertain this, we decided to carry out a small-scale RNA interference (RNAi) screen to evaluate the effect of the down-regulation of the expression of 5 transmembrane glucose transporters upon *P. berghei* infection (Supplementary Table 1). The screen included all class I GLUT genes (GLUT1-4) as well as GLUT9, a major regulator of influx in HepG2 hepatoma cells (Takanaga et al., 2008).



Huh7 cells stably expressing shRNA sequences targeting the selected genes were constructed to be used throughout the RNAi screen. The screen proceeded in 3 consecutive steps, employing different established methods to measure hepatic infection by *P. berghei*. In the first step of the screen, 3 shRNA sequences were used to silence each of the selected genes and the resulting stable cell lines were infected with luciferase-expressing *P. berghei* sporozoites. Forty-eight h later, the parasite load in these cells was determined by measuring the bioluminescence of cell lysates. Each sequence was used in at least 4 independent experiments and an shRNA targeting SR-BI was employed as a positive control for infection decrease, as this receptor has been implicated in hepatic cell invasion by *P. berghei* parasites (Rodrigues et al., 2008). GLUT1 emerged as the gene whose knock-down (KD) leads to the strongest decrease in infection (Fig. 3A), with GLUT4 and GLUT9 KD yielding a more moderate effect (Fig. 3A). We then carried out the second step of the RNAi screen, where only GLUT1, GLUT4 and GLUT9 were silenced prior to infection with GFP-expressing *P. berghei* parasites. The extent of parasite development was assessed 48 h later by flow cytometry determination of GFP intensity (Prudencio et al., 2008). The results showed that KD of GLUT1 leads to the strongest impairment of parasite development among the 3 genes assessed (Fig. 3B). Therefore, GLUT1 was selected for the final confirmation screening step using the three different shRNA sequences targeting the *GLUT1* gene and assessing parasite development by immunofluorescence microscopy (Fig. 3C). Interestingly, the imaging data demonstrated that parasite size correlates with the mRNA levels of GLUT1, suggesting that GLUT1-mediated glucose uptake is required for the parasite's development (Fig. 3C and Supplementary Table 1).

To further establish a link between the uptake of glucose by GLUT1 and *Plasmodium* infection, we assessed the difference in 2-NBDG uptake between infected and non-infected Huh7 cells expressing the same three *GLUT1*-targeting shRNA sequences as above. This difference was drastically reduced in the GLUT1-KD stable cell lines, relative to scramble-transduced control cells, a decrease that is proportional to the extent of the down-modulation



of GLUT1 (Fig. 3D). As an additional control, we assessed 2-NBDG uptake following KD of GLUT2, whose expression by Huh7 cells we confirmed ( $2.6\pm 0.8$ -fold lower than GLUT1), and can therefore contribute to glucose uptake by these cells. Nevertheless, our results showed that, contrary to GLUT1 the down-modulation of the expression of GLUT2 does not affect 2-NBDG uptake by cells containing developing parasites (Fig. 3D). We also confirmed that GLUT1 KD does not affect glucose uptake by non-infected cells except for a small decrease in cells where the down-modulation of GLUT1 expression is most pronounced (Fig. S5).

Finally, we assessed infection, as well as 2-NBDG uptake by infected cells, in the presence of WZB117, a specific inhibitor of GLUT1-mediated glucose transport (Liu et al., 2012). Our results provide a chemical validation of the genetic approach (Fig. 3), as they show a clear dose-dependent effect of WZB117 on overall *P. berghei* infection of Huh7 cells (Fig. 4A), on parasite development (Fig. 4B), and on the difference in 2-NBDG uptake between cells infected with developing parasites and non-infected cells (Fig. 4C). Additionally, we showed that *P. berghei* infection of mouse primary hepatocytes is significantly impaired by the addition of 100  $\mu$ M WZB117 to the cell culture medium (Fig. S6). Most importantly, intra-peritoneal administration of WZB117 to mouse models of *Plasmodium* infection significantly decreases the parasite load in the livers of *P. berghei*-infected mice relative to vehicle-treated control animals (Fig. 4D). Immunofluorescence microscopy analysis of liver sections of these mice (Fig. 4E) revealed a significant decrease in parasite areas (Fig. 4F) and numbers (Fig. 4G) in WZB117-treated animals. These results indicate that GLUT1 inhibition by WZB117 leads to the inhibition of the hepatic parasite's development, as well as to its decreased survival *in vivo*.

## **GLUT1 expression is not enhanced in *Plasmodium*-infected cells**

Having established a role for GLUT1 in the specific uptake of glucose by infected cells, we hypothesized that *Plasmodium* infection might lead to an increase in the expression of that transporter. To address this, we compared GLUT1 expression levels in non-infected and infected Huh7 cells at 6, 30 and 48 hpi with GFP-expressing *P. berghei* parasites. Following the separation of infected and non-infected cells by fluorescence-activated cell sorting (FACS) (Albuquerque et al., 2009), cells were analyzed by quantitative real-time PCR, employing GLUT1-specific primers (Supplementary Table 2). The data showed no significant differences in GLUT1 expression between infected and non-infected cells, at the selected time points (Fig. S7). As such, we concluded that the increase in GLUT1-mediated glucose uptake by *Plasmodium*-infected cells does not result from an infection-induced enhancement of the mRNA expression of this transporter.

## ***P. berghei* development leads to cytoplasmic ATP depletion**

It has been shown that GLUT1 has a cytoplasmic pocket that is postulated to allow the binding of ATP, which in turn induces conformational changes that inhibit GLUT1-mediated glucose transport (Cloherty et al., 1996; Levine et al., 1998). This modulation of GLUT1 conformation by ATP is counteracted by the binding of AMP and ADP to the same site in GLUT1 (Blodgett et al., 2007). We thus wondered whether the observed increase in glucose uptake by *Plasmodium*-infected cells would correlate to a decrease in ATP levels in these cells. To investigate this, we employed a fluorescence resonance energy transfer (FRET)-based indicator for ATP (ATeam), composed of the  $\epsilon$  subunit of the bacterial  $F_0F_1$ -ATP synthase sandwiched between the cyan- and Venus fluorescent proteins (CFP and Venus, respectively), which enables measurement of intracellular ATP levels as a function of the Venus/CFP ratio (Imamura et al., 2009). Using RFP-expressing *P. berghei* parasites and live fluorescence microscopy, we specifically monitored ATP levels in non-infected and infected

cells at 30 and 48 hpi. Interestingly, we observed that the ATP levels of infected cells were significantly lower than those of naïve cells (Fig. 5A). In agreement with the results in Figure 2A, which show that non-infected cells take up more 2-NBDG than naïve cells, we also detected a smaller but significant ATP reduction in the non-infected cells (Fig. 5A), when compared with naïve cells. These data indicate that liver stage parasite development results in a decrease in the ATP available inside the host cell, presumably leading to the well-described ATP/ADP/AMP-driven conformational changes in GLUT1 (Blodgett et al., 2007; Cloherty et al., 1996; Levine et al., 1998).

### ***P. berghei* infection leads to GLUT1 translocation to the plasma membrane**

In order to compare the amount of GLUT1 present on the surface of infected and non-infected cells, Huh7 cells were infected with RFP-expressing *P. berghei* parasites and incubated with the fusion peptide H<sub>RBD</sub>-EGFP (consisting of the Receptor Binding Domain of the human T cell leukemia virus fused to enhanced green fluorescent protein), which has been shown to specifically bind to GLUT1 at the extracellular surface of the cell plasma membrane (Kinet et al., 2007; Manel et al., 2003). CoCl<sub>2</sub> was used as a positive control as it has been shown to enhance GLUT1 translocation to the plasma membrane (Koseoglu and Beigi, 1999). Flow cytometry analysis showed a similar increase in the amount of plasma membrane-located GLUT1 in both non-infected and infected cells at 30 hpi, relative to naïve cells (Fig. 5B). However, at 48 hpi, the amount of GLUT1 at the cell surface is specifically and significantly enhanced in infected cells (Fig. 5B), an increase whose magnitude is consistent of that observed upon stimulation of RAT2 cells by 12-O-tetradecanoyl-phorbol-13-acetate (TPA) (Lee et al., 2015). Our results suggest that an enhancement of GLUT1 availability at the plasma membrane facilitates the uptake of glucose required for the later stages of parasite development, the time at which there is a dramatic increase in glucose uptake (Fig. 2A). Overall, our data support the notion that increased glucose uptake by *P.*

*berghei*-infected hepatic cells is mediated by GLUT1, which becomes activated by the ATP depletion and translocated into the membrane of the infected cell (Fig. 6).

## Discussion

*Plasmodium* parasites require large amounts of nutrients for their extensive replication inside liver cells. Here, we show that glucose uptake is significantly increased in infected hepatic cells, through the enhanced action and translocation of the GLUT1 membrane transporter.

Glucose is ubiquitously used as the common currency of metabolism, and the ability to transport this hexose across the plasma membrane is a feature of nearly all cells (Mueckler, 1994). Although GLUT2 is the major glucose transporter of hepatocytes, where it is involved in glucose uptake and release in the fed and fasted states, respectively (Mueckler and Thorens, 2013; Thorens et al., 1990), GLUT1 is also present in the liver (Karim et al., 2012), where it is transcribed and expressed by both periportal and perivenular hepatocytes. Of note, GLUT2 has a high capacity but a low affinity for glucose, with a  $K_m$  value (the concentration of glucose at which transport is half of its maximal value) in the order of 17 mM (Uldry et al., 2002). GLUT1, on the other hand, has a higher affinity for glucose with a  $K_m$  value of ~3 mM (Uldry et al., 2002), which is closer to that of PfHT (~1 mM, (Woodrow et al., 2000)). Thus, it is tempting to speculate that GLUT1 is better matched to supply glucose to the parasite, especially if local glucose concentrations decrease towards the  $K_m$  of GLUT1. Interestingly, despite being expressed by all hepatocytes, membrane localization of GLUT1 under basal conditions is restricted to hepatocytes proximal to the hepatic venule (Bilir et al., 1993; Karim et al., 2012; Mueckler and Thorens, 2013; Tal et al., 1990; Thorens et al., 1990). Since the liver cell plate is perfused unidirectionally from portal to hepatic venule, the concentration of several substrates, such as oxygen and glucose, decreases as blood moves closer to the latter (Bilir et al., 1993). This is consistent with the observation

that GLUT1 expression is enhanced by a decrease in circulating glucose levels (Simpson et al., 1999) as well as by hypoxia (Ebert et al., 1995). Importantly, it has recently been shown that hypoxia enhances liver stage infection by malaria parasites, an effect that is also observed following treatment with an activator of hypoxia inducer factor (HIF)-1 $\alpha$  or with the hypoxia mimetic CoCl<sub>2</sub> (Ng et al., 2014). Interestingly, increased HIF-1 $\alpha$  levels have been shown to upregulate expression of GLUT1 (Chen et al., 2001) and CoCl<sub>2</sub> has been shown to enhance GLUT1 translocation to the plasma membrane ((Koseoglu and Beigi, 1999) and Fig. 5B). In this context, our results suggest that GLUT1-mediated glucose transport may provide a crucial link to explain the observed preferential infectivity of hypoxic liver cells by *Plasmodium* parasites.

The data presented here is consistent with our proposed model (Fig. 6), in which the extensive replication of liver stage parasites promotes the depletion of intracellular glucose and, consequently, of ATP. This effect is compensated for by an increase in glucose uptake that results from (i) the likely activation of GLUT1 transporters at the plasma membrane via AMP-dependent conformational changes and (ii) from GLUT1 translocation to the plasma membrane, particularly towards the end of parasite development. The significant increase in glucose uptake by infected cells likely leads to a decrease in glucose availability in the vicinity of infected cells, which may result in a similar, albeit weaker, response by neighboring non-infected cells. In fact, these cells also display a small decrease in ATP levels, as well as some, although not statistically significant, GLUT1 translocation to the membrane (Fig. 5A,B), and a slight increase in glucose uptake from 30 hpi onwards (Fig. 2A). A question that remains open is which mechanism(s) is(are) involved in regulating GLUT1 activation and translocation into the membrane of the infected cell. It has previously been shown that enhanced glucose transport by GLUT1 transporters preexisting in the plasma membrane is associated with stimulation of AMP-activated protein kinase (AMPK) activity (Abbud et al., 2000; Barnes et al., 2002). On the other hand, it has been shown that GLUT1 translocation to the plasma membrane may be triggered by insulin (Egert et al.,

1999), in a phosphoinositide 3-kinase (PI3K)-dependent fashion (Egert et al., 1999; Perrini et al., 2004). Intriguingly, neither parasite development nor glucose uptake by *P. berghei*-infected cells seem to be affected by the down-regulation of either the  $\alpha 1$  and the  $\alpha 2$  subunits of AMPK (Fig. S8). Likewise, addition of insulin or inhibition of PI3K with Wortmannin (Hsu and Yang, 2014; Nagai et al., 2011) do not impact infection or glucose uptake by infected cells (Fig. S9). Very recently, phosphorylation of GLUT1 by protein kinase C (PKC) has been shown to lead to the rapid increase in glucose uptake and enhanced cell surface localization of GLUT1 induced by TPA (Lee et al., 2015). Whether a similar mechanism may be at play during *P. berghei* infection of hepatic cells is currently under investigation.

Despite the fact that, under basal conditions, GLUT1 is only localized at the membrane of perivenous hepatocytes, we showed that *in vivo* chemical inhibition of GLUT1-mediated glucose uptake has a substantial impact on the number of *P. berghei*-infected hepatocytes. There are two possible explanations for this observation. The first is that the inhibitor only hinders GLUT1-mediated glucose uptake on perivenous hepatocytes and, therefore, only has an effect on the parasites inside these hepatocytes. Alternatively, in accordance with our model, the replication of the parasite inside periportal hepatocytes could lead to a depletion of the cellular glucose pool and trigger the translocation of GLUT1 present in the endoplasmic reticulum (ER), Golgi and endosomal compartments to the plasma membrane, thereby increasing the percentage of infected hepatocytes with GLUT1 at the plasma membrane and, therefore, the number of cells targeted by the inhibitor.

The liver displays the capacity to remove 30–40% of the glucose presented to it following glucose ingestion and therefore must be considered a significant site of postprandial glucose removal (Pagliassotti and Cherrington, 1992). Interestingly, a recent case-control study in Ghana found that patients with type 2 diabetes mellitus had a 46% increased risk for infection with *P. falciparum* (Danquah et al., 2010). The authors of this study offer a number of possible explanations for this observation, including impaired immune responses, an

increased number of infectious mosquito bites, and enhanced parasite growth in the blood of type 2 diabetes mellitus patients (Danquah et al., 2010). Thus, our results suggest the possibility that the elevated amounts of circulating glucose displayed by diabetic patients may also promote parasite development in the liver, thereby contributing to the increased risk of appearance of clinically relevant malaria. Further investigation of the link between diabetes and malaria is clearly warranted by the fact that sub-Saharan Africa currently faces the world's highest increase in type 2 diabetes mellitus (Wild et al., 2004).

To the best of our knowledge, this is the first report that addresses the role of glucose during hepatic infection by *Plasmodium* from a molecular point of view. It identifies GLUT1 as a druggable target and a major player in glucose uptake by infected cells and shows that its function as a glucose transporter is modulated in cells containing replicating parasites.

These findings contribute to an understanding of how hepatic host cells meet the energy demands imposed by the parasite's huge replication rate. Such an understanding may help identify strategies to control energy production by infected cells and thereby limit parasite development and survival.

## **Experimental Procedures**

### **Chemicals**

RPMI 1640, PBS pH 7.4, trypsin, fetal bovine serum (FBS), non-essential amino acids, penicillin/streptomycin, glutamine, HEPES pH 7, OptiMEM and Lipofectamine RNAiMAX were purchased from Gibco/Invitrogen. All other chemicals were obtained from Sigma, unless otherwise specified.

### **Cells**

Huh7 cells, a human hepatoma cell line, were cultured in RPMI 1640 medium supplemented with 10% v/v FBS, 0.1 mM non-essential amino acids, 50 µg/ml penicillin/streptomycin, 2



mM glutamine and 1 mM HEPES (final concentrations), pH 7 and maintained at 37°C with 5% CO<sub>2</sub>. Mouse primary hepatocytes were cultured in William's E medium supplemented with 4% FBS, 2 mM glutamine, 1 mM HEPES and 50 µg/ml penicillin/streptomycin, and maintained at 37°C with 5% CO<sub>2</sub>.

### **Mice**

C57BL/6 mice were purchased from Charles River and housed in the rodent facility of Instituto de Medicina Molecular (Lisbon, Portugal). All animal experiments were performed in strict compliance to the guidelines of our institution's animal ethics committee and the Federation of European Laboratory Animal Science Associations (FELASA).

### **Parasites**

Green fluorescent protein (GFP)-, red fluorescent protein (RFP)-, or luciferase-expressing *P. berghei* ANKA sporozoites were dissected in non-supplemented RPMI medium from the salivary glands of infected female *A. stephensi* mosquitoes, bred at Instituto de Medicina Molecular, prior to being employed for *in vitro* and *in vivo* infections (Franke-Fayard et al., 2004; Ploemen et al., 2009).

### **Overall *in vitro* infection by luminescence**

Overall hepatic infection was determined by measuring the luminescence intensity in Huh7 cells or mouse primary hepatocytes infected with a firefly luciferase-expressing *P. berghei* line, as previously described (Ploemen et al., 2009). Briefly, Huh7 cells or mouse primary hepatocytes (1.0x10<sup>4</sup> and 2.0x10<sup>4</sup> per well, respectively) were seeded in 96-well plates the day before infection. Sporozoite addition was followed by centrifugation at 1800xg for 5 min and the medium was replaced approximately 2 hpi by the appropriate medium. Parasite infection load was measured 48 hpi by a bioluminescence assay (Biotium) using a multiplate

reader Infinite M200 (Tecan). The effect of the different treatments on cell viability was assessed by the CellTiter-Blue assay (Promega) according to the manufacturer's protocol.

### **Quantification of *P. berghei* invasion and development by flow cytometry**

Invasion of hepatoma cells and intracellular parasite development were assessed by determining the percentage of GFP<sup>+</sup> cells 2 hpi with a GFP-expressing *P. berghei* line and by measuring the intensity of the GFP signal of the infected cells 48 hpi, respectively, as previously described (Prudencio et al., 2008). Huh7 cells ( $5.0 \times 10^4$  per well) were seeded in 24-well plates the day before infection. The medium was replaced by the appropriate medium 1 h prior or 2 hpi, for invasion and development quantification, respectively. Cells were then collected for flow cytometry analysis at 2 h or 48 hpi, respectively, and analyzed on a Becton Dickinson FACScalibur. Data acquisition and analysis were carried out using the CELLQuest (version 3.1.1 f1, Becton Dickinson) and FlowJo (version 6.4.7, FlowJo) software packages, respectively.

### **Immunofluorescence imaging of *P. berghei* in Huh7 cells**

For immunofluorescence microscopy analyses, cells were seeded on glass coverslips in 24-well plates and infected with sporozoites as described above. Forty-eight hpi, cells were rinsed with 1x PBS and fixed with 4% v/v paraformaldehyde (PFA; Santa Cruz Biotechnology) for 20 min at room temperature and stored at 4°C in PBS 1x until being stained. Cells were incubated with the permeabilization/blocking solution (0.1% v/v Triton X-100, 1% w/v bovine serum albumin (BSA) in 1x PBS) for 30 min at room temperature. Parasites were stained with a parasite specific anti-Heat Shock Protein 70 (Hsp70) antibody (2E6; dilution 1:100) and an anti-UIS4 antibody (dilution 1:1000) for 1 h at room temperature, followed by three washes with permeabilization/blocking solution. Cells were further incubated in a 1:400 dilution of anti-mouse Alexa-Fluor 488 (Jackson ImmunoResearch Laboratories) or anti-goat Alexa-Fluor 568 (Life Technologies) secondary antibodies in the presence of a 1:1000 dilution of Hoechst 33342 (Invitrogen) for nuclei staining. An additional

three washes were carried out with permeabilization/blocking solution. Coverslips were mounted on microscope slides with Fluoromount (SouthernBiotech). Confocal images were acquired using a Zeiss LSM 710 confocal microscope. Widefield images for size determination were acquired in a Zeiss Axiovert 200M microscope. Images were processed with ImageJ software (version 1.47).

### **Quantification of glucose uptake by flow cytometry and live microscopy**

Glucose uptake into Huh7 cells was quantified by flow cytometry using a fluorescent D-glucose derivative, 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose (2-NBDG; Molecular Probes, Life Technologies) as a tracer (O'Neil et al., 2005; Yamada et al., 2007). Briefly, Huh7 cells ( $1.0 \times 10^4$  per well) were seeded in 96-well plates the day before infection with RFP-expressing *P. berghei* sporozoites. At several time points after infection, the medium was replaced by RPMI without glucose supplemented with 1 mM of glucose and 0.1 mM of 2-NBDG, and cells were incubated for 5 min at 37°C to allow uptake (O'Neil et al., 2005). Cells were then collected for flow cytometry analysis and analyzed on a BD LSR Fortessa flow cytometer with the DIVA software (version 6.2). Analysis was carried out using the FlowJo software (version 6.4.7, FlowJo). For live microscopy, Huh7 cells ( $2.0 \times 10^5$ ) were seeded on glass bottom microwell dishes (MatTek) the day before infection with RFP-expressing *P. berghei* sporozoites. At 48 hpi, the medium was replaced by RPMI without glucose supplemented with 1 mM of glucose and 0.1 mM of 2-NBDG, and cells were incubated for 30 min at 37°C. Cells were then visualized on a 3i Marianas SDC microscope and the acquired images were processed with ImageJ software (version 1.47).

### **Temperature shocks and ethanol-induced oxidative stress**

Huh7 cells ( $1.0 \times 10^4$  per well) were seeded in 96-well plates and incubated at 37°C for 48 h. To induce a mild cold stress response, one of the plates was incubated at 27°C for 1 h (Fujita, 1999). To induce a heat stress response, another plate was incubated at 43°C for 1 h (Sun et al., 2015). A control plate was maintained at 37°C. Ethanol-induced oxidative

stress was attained by incubating Huh7 cells in complete RPMI with 50 mM of ethanol for 24 h (Nourissat et al., 2008; Sergent et al., 2005). Following temperature shocks and ethanol-induced oxidative stress, the uptake of 2-NBDG by the cells was determined as previously described.

### **Viral infection-induced stress**

Huh7 cells ( $5.0 \times 10^4$  per well) were seeded in 24-well plates and, on the following day, were infected with 0.01, 1 and 5 plaque-forming units (PFU)/cell of wild type murine gammaherpesvirus 68 (MHV-68) or YFP-expressing MHV-68 (MHV-68-YFP) (Collins et al., 2009). Twenty-four h later, the uptake of 2-NBDG by the MHV-68-infected cells was determined as previously described. The percentage of MHV-68-infected cells in each condition was estimated by determining the percentage of YFP<sup>+</sup> cells after infection with the same amount of PFU/cell of MHV-68-YFP.

### **Assessment of the impact of glucose transporters on *Plasmodium* infection by RNA interference (RNAi)**

Down-modulation of the genes encoding selected glucose transporters employed short hairpin RNAs (shRNAs). All shRNAs were purchased from the MISSION TRC library (Sigma) in the form of bacterial glycerol stocks which were grown to obtain the purified plasmids. Each gene was targeted by using three distinct shRNAs, used individually (Supplementary Table 1). For the lentiviral production, HEK 293FT cells ( $2.0 \times 10^4$  per well) were seeded in 96-well plates. On the following day, cells were transfected with the packaging vectors and each individual shRNA plasmid in a final concentration of 100 ng/well using the FuGENE 6 reagent (Promega), according to the manufacturer's instructions. The lentiviral particles were collected in the supernatant of these cells approximately 60 h after transfection and stored at  $-80^\circ\text{C}$ . For the transduction of Huh7 cells and subsequent generation of cell lines with stable knockdown of the genes of interest, cells ( $1.0 \times 10^5$  per

well) were seeded in 12-well plates. On the following day, the medium was replaced by 400  $\mu$ L of supplemented RPMI with 8  $\mu$ g/mL of polybrene, on top of which were added 100  $\mu$ L of lentiviral particles-containing supernatant (approximate MOI of 1:1) and the plates were centrifuged for 30 min at 1200xg and 37°C. Twenty-four h after transduction, the medium was replaced by supplemented RPMI with 5  $\mu$ g/mL of puromycin (Calbiochem) for the selection of the transduced cells which were allowed to grow for at least a week before being used for infections with luciferase- or GFP-expressing *P. berghei* sporozoites to determine overall infection and parasite development by luminescence, flow cytometry and microscopy, as described above. Cells transduced with lentiviral particles carrying a negative control shRNA (SHC002) not targeting any annotated gene in the human genome were used as negative control. A stable cell line with the knockdown of SR-BI was used as positive control in the luminescence assays (Rodrigues et al., 2008). The knockdown efficiency of each shRNA sequence (Supplementary Table 1) was assessed by quantitative PCR (qPCR) with specific primers for each gene (Supplementary Table 2).

### **siRNA transfection**

$4.0 \times 10^4$  Huh7 cells were reverse-transfected with 30 nM of target specific (human AMPK  $\alpha$ 1: ref. L-005027-00-0005; human AMPK  $\alpha$ 2: ref. L-005361-00-0005) or control siRNA sequence pools (ON-TARGETplus SMARTpool, Dharmacon), using Lipofectamine RNAiMAX (Gibco/Invitrogen) according to the manufacturer's instructions. Twenty-four h after transfection, the cells were infected with  $3.0 \times 10^4$  GFP-expressing *P. berghei* sporozoites. Cells were collected for flow cytometry analysis at 48 hpi and analyzed on a BD LSR Fortessa flow cytometer with the DIVA software (version 6.2). Analysis was carried out using the FlowJo software (version 6.4.7, FlowJo). The efficiency of knockdown was assessed with specific primers by qPCR (Supplementary Table 2).

## **FACS-sorting of *P. berghei*-infected and non-infected Huh7 cells**

Huh7 cells ( $1.0 \times 10^5$  per well) were seeded in 24-well plates and infected 24 h later with  $1.0 \times 10^5$  GFP-expressing *P. berghei* sporozoites. Cells were collected at 2 hpi and FACS-sorted on a BD FACSAria III Cell Sorter (BD Biosciences). Non-infected and GFP-expressing *P. berghei*-infected cells were gated on the basis of their different fluorescence intensity, as previously established, and collected simultaneously (Albuquerque et al., 2009; Prudencio et al., 2008). Immediately after FACS-sorting, both infected and non-infected cells were washed and seeded in 24-well plates at a density of  $1.5 \times 10^5$  per well. Infected cells were diluted 1:1 with non-infected cells to allow replicates. Cells were then incubated until collection at 30 or 48 hpi. In the case of the 6 hpi time point, *P. berghei*-infected cells were FACS-sorted at this time, diluted 1:1 with non-infected cells, pelleted, snap-frozen and stored until RNA extraction.

## **RNA extraction, cDNA synthesis and qPCR**

RNA was extracted from cultured cells using the High Pure RNA Isolation kit (Roche) according to the manufacturer's instructions. The amount of RNA in each sample was assessed with a NanoDrop® ND-1000 spectrophotometer.

Complementary DNA (cDNA) was synthesized from 1 µg of RNA using the Roche cDNA synthesis kit, according to the manufacturer's instructions. The cDNA was synthesized employing the following thermocycling parameters: 25°C for 10 min, 55°C for 30 min, and 85°C for 5 min. qPCR reaction was performed in a total volume of 20 µL in a ABI Prism 7500 Fast system (Applied Biosystems) using the iTaq™ Universal SYBR® Green kit (BioRad) as follows: 50°C for 2 min, 95°C for 10 min, 40 cycles at 95°C for 15 sec and 60°C for 1 min, melting stage was done at 95°C for 15 sec, 60°C for 1 min, and 95°C for 30 sec. Primers for hypoxanthine-guanine phosphoribosyltransferase (Hprt), a well-established housekeeping gene, were used for normalization in all experiments (Supplementary Table 2). The delta-delta CT relative quantification method was used for analysis of qPCR results.

### ***In vivo* WZB117 treatment, *P. berghei* sporozoite infection and quantification of parasite liver load by qRT-PCR**

Six weeks old male C57BL/6 mice were injected intraperitoneally (i.p.) with 10 mg/kg of WZB117 in PBS/DMSO 1:1 (v/v), or with vehicle alone, immediately before intravenous (i.v.) injection of  $3.0 \times 10^4$  GFP-expressing *P. berghei* sporozoites (Liu et al., 2012). The administration of the drug or vehicle was repeated at 15 and 30 hpi and the livers were collected at 44 hpi and homogenized in 3 mL of denaturing solution (4 M guanidine thiocyanate; 25 mM sodium citrate pH 7, 0.5% w/v *N*-lauroylsarcosine and v/v 0.7%  $\beta$  mercaptoethanol in DEPC-treated water). Total RNA was extracted from the livers with the NZY Total RNA Isolation Kit (NZYTech), according to the manufacturer's protocol, and converted into cDNA as described above. Parasite load was quantified by qPCR using primers specific to *P. berghei* 18S RNA (Supplementary Table 2). Mouse *Hprt* expression was used for normalization.

### **Immunohistochemical staining of liver sections**

For microscopy, PFA-fixed liver lobes were cut in 50  $\mu$ m sections and were incubated in permeabilization/blocking solution (1% w/v BSA, 0.5% v/v Triton-X100 in PBS) at room temperature for 1 h, followed by a 2 h incubation at room temperature with an anti-UIS4 antibody (dilution 1:500). Slices were further incubated in a 1:300 dilution of anti-GFP-Alexa488 antibody (Invitrogen) and anti-goat Alexa-Fluor 568 (Invitrogen) in the presence of a 1:1000 dilution of Hoechst 33342 (Invitrogen) and a 1:100 dilution of Phalloidin-660 (Invitrogen) for actin staining for 1 h. After washing, slices were mounted on microscope slides with Fluoromount (SouthernBiotech). Images were acquired and processed as previously described.

### **Determination of ATP levels inside single cells by live microscopy**

Huh7 cells ( $2.0 \times 10^5$  cells) were seeded on glass bottom microwell dishes (MatTek). The following day, 1  $\mu$ g of ATeam plasmid carrying the ATP indicator (pRSET-AT1.03) was



transfected into the cells with FuGENE 6 (Roche) according to the manufacturer's instructions (Imamura et al., 2009). Twenty-four h after transfection, cells were infected with RFP-expressing *P. berghei* sporozoites and, at selected time points of infection, were visualized at 37°C on a Zeiss LSM 710 confocal microscope as described previously (Ando et al., 2012). Huh7 cells incubated for 6 h in RPMI without glucose with 10 mM of galactose and 10 µM of Oligomycin were used as positive controls for ATP depletion. Image analysis was performed using the ImageJ software (version 1.47). The Venus/CFP emission ratio was calculated for each cell dividing its mean intensity in the Venus channel by the mean intensity in the CFP channel.

### **Quantification of GLUT1 at the plasma membrane of infected cells by flow cytometry**

Huh7 cells ( $1.0 \times 10^4$  per well) were seeded in 96-well plates and were infected with RFP-expressing *P. berghei* sporozoites on the following day. Thirty and 48 hpi, cells were detached using PBS containing 1 mM EDTA, centrifuged at 4°C, and the cell pellet was resuspended in cold RPMI containing 10% v/v FBS. Cells were further incubated with supernatant obtained from 293T cells transfected with a vector encoding the H<sub>RBD</sub>-EGFP fusion protein (dilution 1:50 in PBS with 2% v/v FBS) (Kinet et al., 2007; Manel et al., 2003). Following incubation at 37°C for 30 min, cells were harvested by centrifugation, washed one time with PBS with 2% v/v FBS, resuspended in the same buffer and analyzed with a BD LSR Fortessa cytometer with the DIVA software (version 6.2). Huh7 cells incubated overnight in complete RPMI with 250 µM of cobalt (II) chloride (CoCl<sub>2</sub>) were used as positive controls (Koseoglu and Beigi, 1999). Analysis was carried out using the FlowJo software (version 6.4.7, FlowJo).

### **Statistical Analyses**

Statistical analyses were performed using the GraphPad Prism 5 software. One-way ANOVA, Two-way ANOVA, Chi-Square, Mann-Whitney U test or Student's t-test were used for significance of differences observed, as indicated in each figure. ns – not significant, \*  $p < 0.05$ , \*\*  $p < 0.01$  and \*\*\*  $p < 0.001$ .

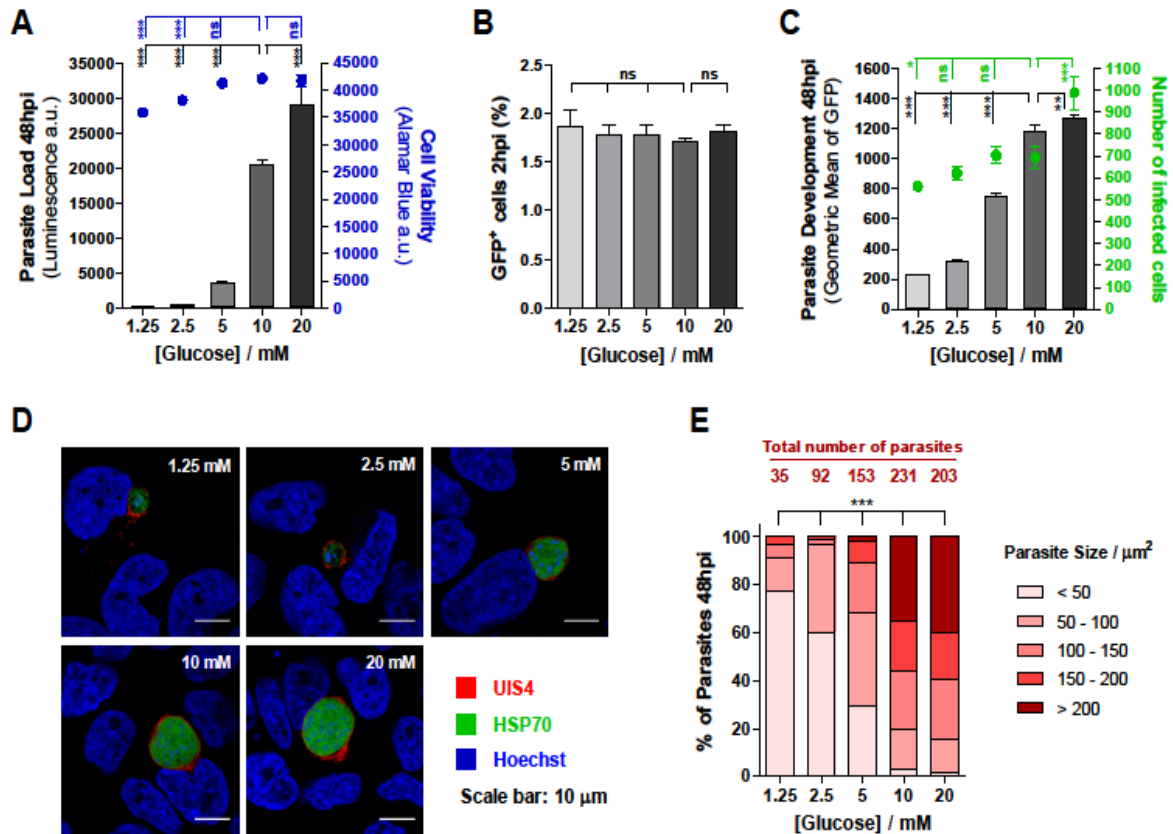
**Supplementary Table 1 - List of shRNA sequences used, with the corresponding knockdowns.**

Protein	Gene	Gene ID	Sequence	Clone ID	Oligonucleotide Sequence	Knockdown
GLUT1	SLC2A1	NM_006516	#01	TRCN0000043583	CCGGGCCACACTATTACCATGAGAACTCGAGTTCTCATGGTAATAGTGTGGCTTTTTG	90%
			#02	TRCN0000043584	CCGGGCGGAATTCAATGCTGATGATCTCGAGATCATCAGCATTGAATCCGCTTTTTG	80%
			#03	TRCN0000043585	CCGGCTTCTATTACTCCACGAGCATCTCGAGATGCTCGTGGAGTAATAGAAGTTTTG	40%
GLUT2	SLC2A2	NM_000340	#01	TRCN0000043598	CCGGGCCCAACAATCTCATACTCAATCTCGAGATTGAGTATGAGATTGTGGCTTTTTG	0%
			#02	TRCN0000043600	CCGGGCAAAACATTCTGTCATTAGTTCTCGAGAATAATGACAGAATGTTTGCTTTTTG	45%
			#03	TRCN0000043601	CCGGCGACGTTCTCTCTTTCTAATTCTCGAGAATTAGAAAGAGAGAACGTCGTTTTG	70%
GLUT3	SLC2A3	NM_006931	#01	TRCN0000043614	CCGGCCTGAGAAGATCATAAAGGAACTCGAGTTCCTTTATGATCTTCTCAGGTTTTG	80%
			#02	TRCN0000043615	CCGGAGTAGCTAAGTCGGTTGAAATCTCGAGATTTCAACCGACTTAGCTACTTTTTG	95%
			#03	TRCN0000043617	CCGGCCCAGATCTTTGGTCTGGAATCTCGAGATTCCAGACCAAGATCTGGGTTTTG	60%
GLUT4	SLC2A4	NM_001042	#01	TRCN0000043629	CCGGCCTTCTTAAGAGTACCTGAAACTCGAGTTTCAGGTA CTCTTAAGAAGTTTTG	90%
			#02	TRCN0000043630	CCGGCTTGAGTATTTAGGGCCAGATCTCGAGATCTGGCCCTAAATACTCAAGTTTTG	60%
			#03	TRCN0000043632	CCGGGTGATTGAACAGAGCTACAATCTCGAGATTGTAGCTCTGTTCAATCACTTTTTG	80%
GLUT9	SLC2A9	NM_001001290	#01	TRCN0000043683	CCGGACGTTGGACAATTATGTCAAACCTCGAGTTTGACATAATTGTCCAACGTTTTG	70%
			#02	TRCN0000043684	CCGGCGCTTCATCATGGGCATAGATCTCGAGATCTATGCCCATGATGAAGCGTTTTG	25%
			#03	TRCN0000043685	CCGGCCATACCTGTTTGGAGTGATTCTCGAGAATCACTCCAACAGGTATGGTTTTG	70%
SR-BI	SCARB1	NM_005505		TRCN0000056966	CCGGGCAAGGTTGACTTCTGGCATTCTCGAGAATGCCAGAAGTCAACCTTGCTTTTTG	85%

**Supplementary Table 2 - List of primer sequences.**

<b>Gene</b>	<b>forward primer (5' - 3')</b>	<b>reverse primer (5' - 3')</b>
SLC2A1	CGTCGCCACCCGCGTAC	AACTCCTCGATCACCTTCTGGGG
SLC2A2	AGATAAGGTCCTGGGACCCTGGT	GCTTTTCGGTCATCCAGTGGAACA
SLC2A3	GGACGTGGAGAAAACCTTGCTGCTG	GCCGATTGTAGCAACTGTGATGGCA
SLC2A4	AGACGAGATGCCGTCGGGCT	TGTTCAATCACCTTCTGAGGGGCA
SLC2A9	CAAGAGCTGTGAAAGCCTTCC	ATGGTGACAATCACGGTGACC
SCARB1	AGAATAAGCCCATGACCCTGAA	TGAGCTCAGCAATAATCCGAA
AMPK $\alpha$ 1	AGGAGAGCTATTTGATTATATCTGTAAGAATG	ACACCAGAAAGGATCTGTTGGAA
AMPK $\alpha$ 2	CGGCTCTTTCAGCAGATTCTGT	ATCGGCTATCTTGGCATTTCATG
Pb18S	AAGCATTAAATAAAGCGAATACATCCTTAC	GGAGATTGGTTTTGACGTTTATGTG
HPRT	TTTGCTGACCTGCTGGATTAC	CAAGACATTCTTCCAGTTAAAGTTG

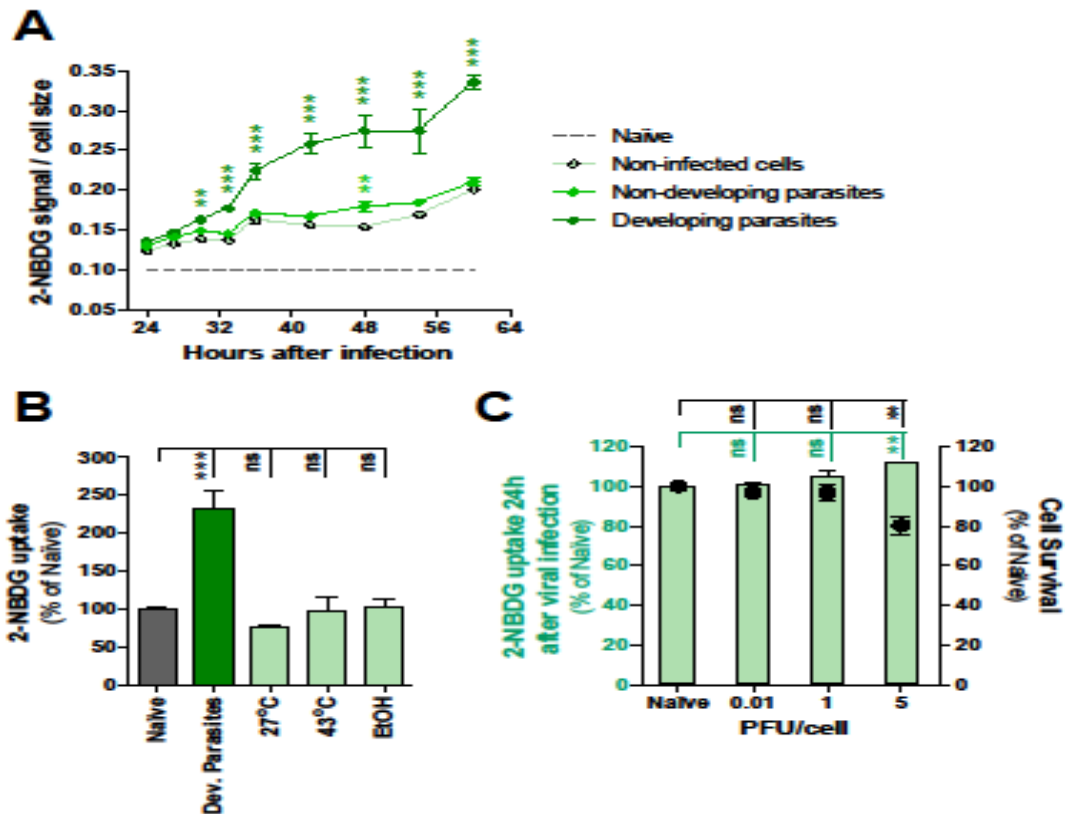
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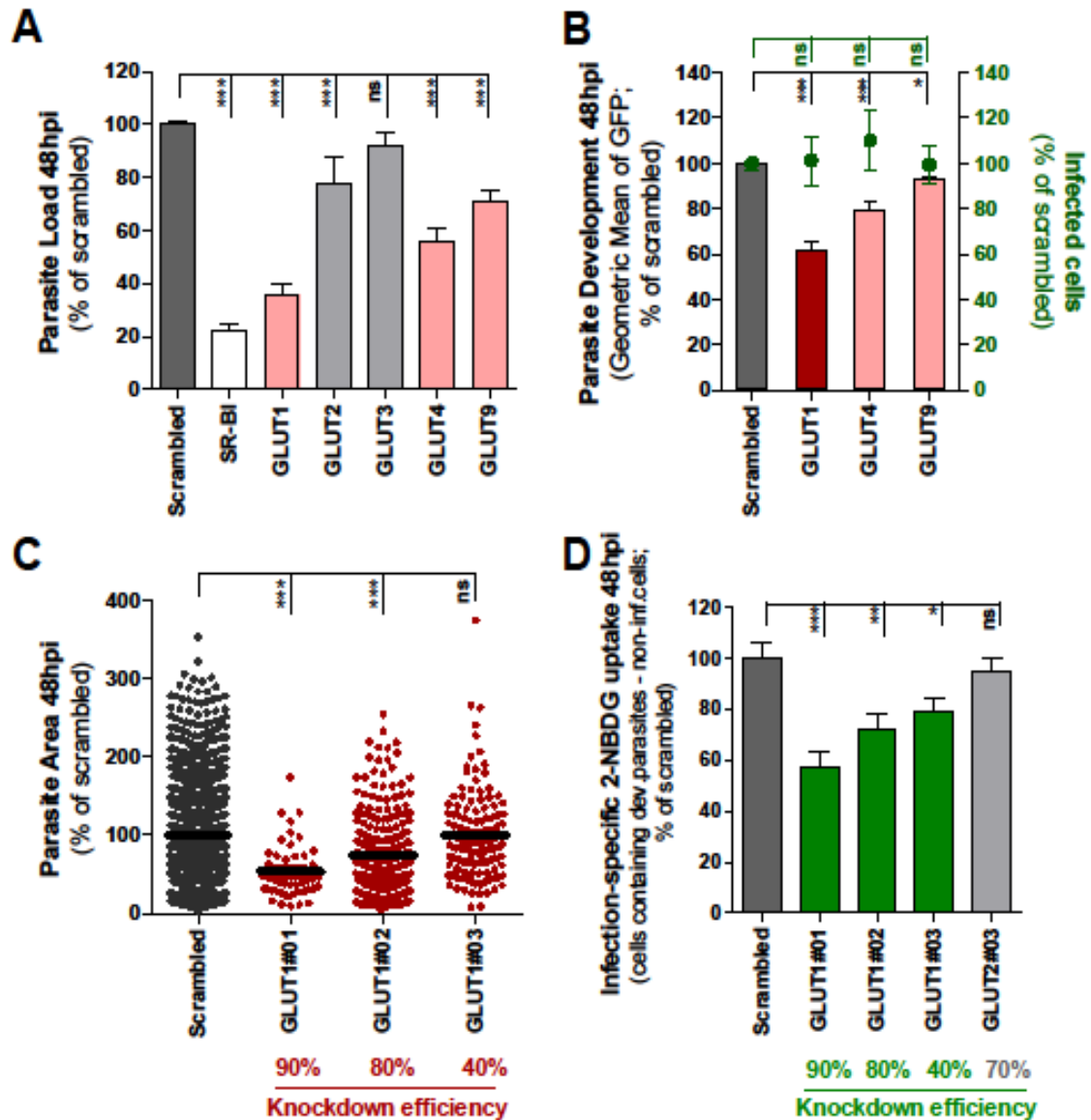
**Figure 1 - Glucose availability impacts *P. berghei* hepatic infection. (A)** Huh7 cells were infected with luciferase-expressing *P. berghei* sporozoites and the culture medium was replaced 2 hpi by medium with different concentrations of glucose. Parasite load (luminescence) and cell viability were assessed after 48 h. Representative experiment out of 2 independent experiments. Error bars represent SD. One-way ANOVA with post-test Dunnett. **(B)** The culture medium of Huh7 cells was replaced by medium with different concentrations of glucose 1 h prior to infection with GFP-expressing sporozoites. Cell invasion was quantified by determining the percentage of GFP<sup>+</sup> cells 2 hpi by flow cytometry. Representative experiment out of 2 independent experiments. Error bars represent SD. One-way ANOVA with post-test Dunnett. **(C)** Huh7 cells were infected with GFP-expressing *P. berghei* sporozoites and the culture medium was replaced 2 hpi by medium with different concentrations of glucose. Parasite development and the total number of infected cells were assessed by flow cytometry by determining the fluorescence intensity and the number of

GFP<sup>+</sup> cells at 48 hpi, respectively. Representative experiment out of 5 independent experiments. Error bars represent SD. One-way ANOVA with post-test Dunnett. **(D)** Representative confocal images of Huh7 cells infected with sporozoites and incubated in medium with different concentrations of glucose for 48 h. Cells were immunostained with anti-UIS4 (red), which localizes to the parasitophorous vacuole membrane, anti-HSP70 (green), which localizes to the parasite's cytoplasm, and Hoechst (blue), a nuclear stain. Scale bar, 10  $\mu$ m. **(E)** For each glucose concentration, the EEFs in the fluorescence microscopy images acquired were counted and their area was determined. EEFs were divided into 5 different size classes. Pool of 2 independent experiments. Chi-square test. ns - not significant, \*  $p < 0.05$ , \*\*  $p < 0.01$  and \*\*\*  $p < 0.001$ .

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**Figure 2 - Glucose uptake is specifically increased in hepatoma cells containing developing *P. berghei* parasites. (A)** 2-NBDG uptake by different cell populations, normalized to cell size, at different time points of infection, assessed by flow cytometry. Error bars represent SD. Two-way ANOVA with post-test Bonferroni. **(B)** 2-NBDG uptake by naïve Huh7 cells, cells containing developing *P. berghei* parasites at 48 hpi, and cells subjected to low-temperature, high-temperature and ethanol-induced oxidative stresses, assessed by flow cytometry. Pool of 2 independent experiments. Error bars represent SD. One-way ANOVA with post-test Dunnett. **(C)** 2-NBDG uptake by naïve Huh7 cells and cells infected with MHV-68 at different MOI, assessed by flow cytometry. Pool of 2 independent experiments. Error bars represent SEM. One-way ANOVA with post-test Dunnett. ns - not significant, \*\*  $p < 0.01$  and \*\*\*  $p < 0.001$ .

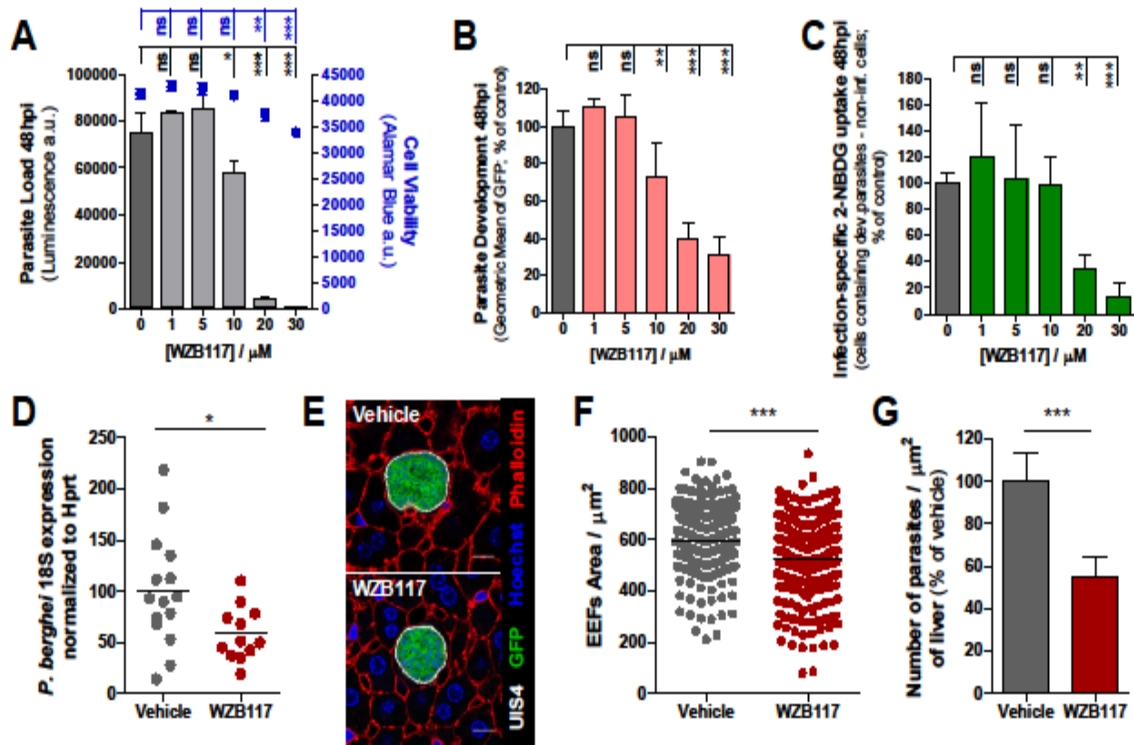


**Figure 3 - GLUT1 knockdown significantly impairs *P. berghei* liver stage development and glucose uptake by *P. berghei*-infected cells. (A)** Huh7 cells with stable knockdown of one of 5 GLUT receptors were infected with luciferase-expressing *P. berghei* sporozoites and parasite load was assessed by luminescence measurement 48 h later. A scrambled shRNA sequence was used as a negative control and a cell line with stable knockdown of SR-BI as positive control. Pool of 4 independent experiments. Error bars represent SEM. **(B)** Huh7 cells with the knockdown of GLUT1, GLUT4 or GLUT9 were infected with GFP-



expressing *P. berghei* sporozoites. The percentage of infected cells and parasite development were assessed by flow cytometry at 48 hpi. Pool of 3 independent experiments. Error bars represent SEM. **(C)** Quantification of the area of the EEFs in each of the 3 different cell lines with GLUT1 knockdown at 48 hpi by immunofluorescence microscopy. The knockdown efficiency of each shRNA sequence is indicated. Pool of 3 independent experiments. **(D)** Difference of 2-NBDG uptake in the several cell lines with GLUT1 knockdown by developing parasites-containing cells and non-infected cells at 48 hpi, assessed by flow cytometry. A cell line with the knockdown of GLUT2 was used as control. Pool of 4 independent experiments. Error bars represent SEM. All panels: one-way ANOVA with post-test Dunnett. ns - not significant, \*  $p < 0.05$ , \*\*  $p < 0.01$  and \*\*\*  $p < 0.001$ .

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**Figure 4 - Chemical inhibition of GLUT1-mediated glucose transport by WZB117**

**affects *P. berghei* hepatic infection *in vitro* (A-C) and *in vivo* (D-G).**

**(A)** Huh7 cells were infected with luciferase-expressing *P. berghei* sporozoites and 2 h later the culture medium was replaced by medium with increasing concentrations of WZB117. Parasite load (luminescence) and cell viability were assessed at 48 hpi. Representative experiment out of 2 independent experiments. Error bars represent SD. One-way ANOVA with post-test Dunnett.

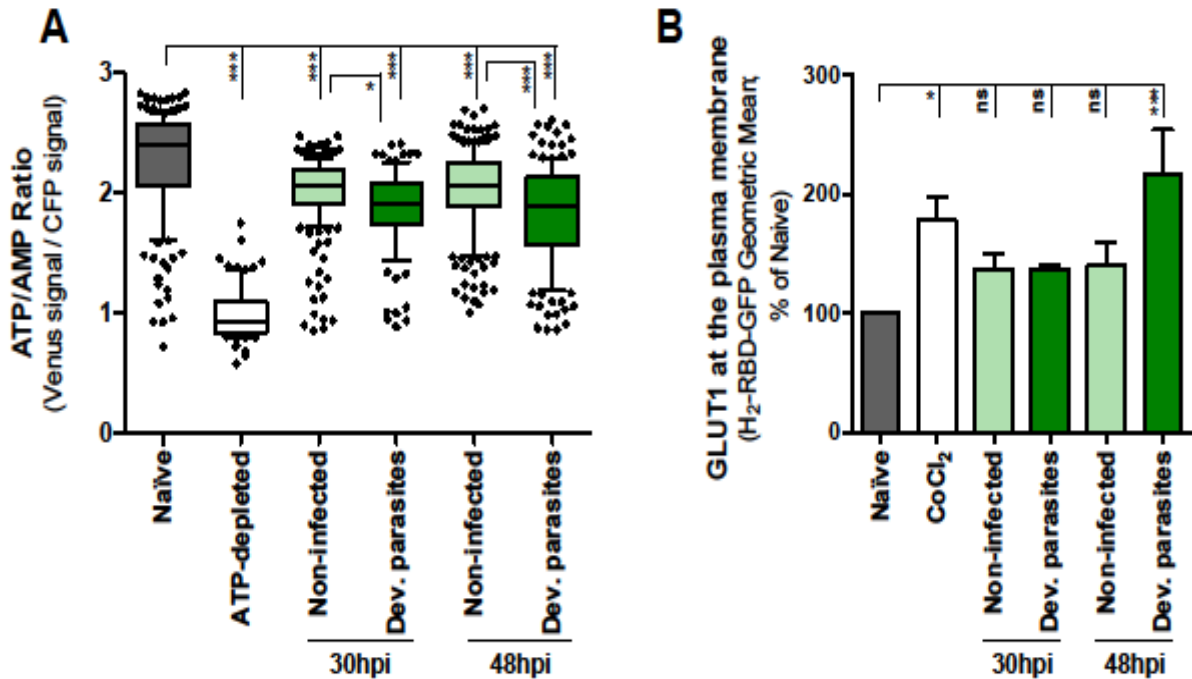
**(B)** Huh7 cells were infected with RFP-expressing *P. berghei* sporozoites and 2 h later the culture medium was replaced by medium with increasing concentrations of WZB117. Parasite development was assessed by flow cytometry at 48 hpi. Pool of 2 independent experiments. Error bars represent SD. One-way ANOVA with post-test Dunnett.

**(C)** 2-NBDG uptake by RFP-expressing *P. berghei*-infected cells at 48 hpi after treatment with increasing concentrations of WZB117, assessed by flow cytometry. Pool of 2 independent experiments. One-way ANOVA with post-test Dunnett.

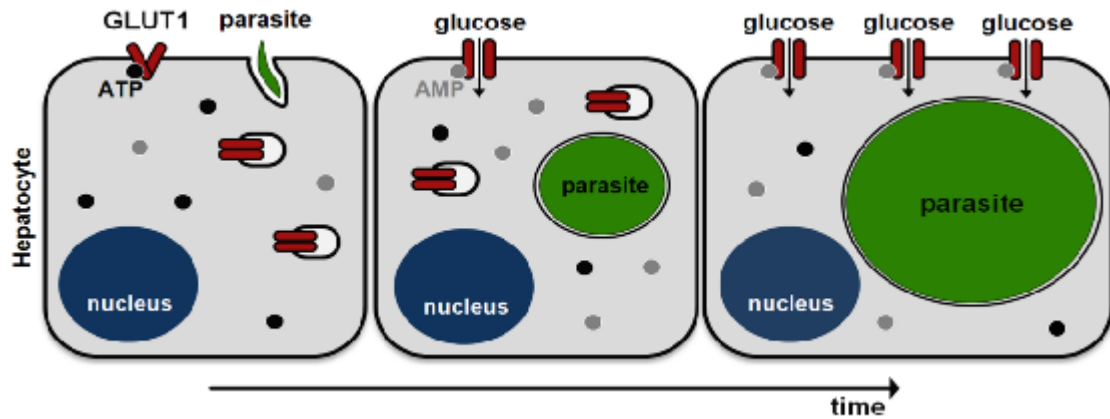
**(D)** C57Bl6/J mice received three doses of 10 mg/kg of WZB117 i.p., the first immediately before infection with *P. berghei* sporozoites and the other two at 15 and 30 hpi. Parasite load was assessed at 44

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hpi by qRT-PCR. Pool of 3 independent experiments. Vehicle: n = 15 mice; WZB117-treated: n = 12 mice. Two-tailed Mann-Whitney test. **(E)** Fifty  $\mu\text{m}$  sections of one liver lobe from one mouse of each experimental group were stained with anti-UIS4 (white), anti-GFP (green), phalloidin (red) and Hoechst (blue). Representative confocal images of *P. berghei* parasites in both experimental groups of mice. Scale bar, 10  $\mu\text{m}$ . **(F)** Area of the parasites in the control (vehicle) or the WZB117-treated mouse, determined by immunofluorescence microscopy. Representative experiment out of 2 independent experiments. Two-tailed Mann-Whitney test. **(G)** Number of parasites per area of liver in control and WZB117-treated mice. Representative experiment out of 2 independent experiments. Error bars represent SD. Two-tailed Mann-Whitney test. ns - not significant, \*  $p < 0.05$ , \*\*  $p < 0.01$  and \*\*\*  $p < 0.001$ .



**Figure 5 – *P. berghei* development inside Huh7 cells results in a decreased ATP/AMP ratio and increased GLUT1 content at the plasma membrane. (A)** Huh7 cells were transfected with an ATP probe, AT1.03, and infected with RFP-expressing *P. berghei* sporozoites. Thirty and 48 hpi, the Venus/CFP emission ratio in the cytoplasm of each individual cell (non-infected and cells containing developing parasites) was calculated from the fluorescent images acquired in both channels. Huh7 cells incubated in RPMI without glucose with 10 mM of galactose and 10  $\mu$ M of Oligomycin were used as positive controls for ATP depletion. Pool of 2 and 3 independent experiments for the 30 h and 48 h time points, respectively. **(B)** Huh7 cells were infected with RFP-expressing sporozoites and, at 30 and 48 hpi, were incubated with the H<sup>RBD</sup>-EGFP peptide that binds specifically to GLUT1 at the surface of the cells and were analysed by flow cytometry. Huh7 cells incubated overnight in complete RPMI with 250  $\mu$ M of CoCl<sub>2</sub> were used as positive controls. Pool of 2 and 4 independent experiments for the 30 h and 48 h time points, respectively. Error bars represent SEM. Both panels: one-way ANOVA with post-test Tukey. ns - not significant, \*  $p < 0.05$  and \*\*\*  $p < 0.001$ .



**Figure 6 - Proposed model of GLUT1-mediated glucose uptake during hepatic**

**infection by *Plasmodium*.** Following the invasion of hepatic cells by *Plasmodium* parasites and the establishment of infection (left), parasites initiate a process of dramatic intracellular replication. Parasite development reduces the amount of host intracellular glucose, leading to depletion of the ATP pool and binding of ADP/AMP to GLUT1 transporters at the plasma membrane. This results in their activation (middle). At later stages of infection, GLUT1 transporters present in the host cell's cytoplasmic compartments are translocated to the cell membrane, leading to further enhancement of glucose uptake (right).

### **Supplementary Figure 1 - Glucose availability impacts *P. berghei* infection ex vivo. (A)**

Mouse primary hepatocytes were infected with luciferase-expressing *P. berghei* sporozoites and the culture medium was replaced 2 hpi by medium with different concentrations of glucose. Parasite load (luminescence) and cell viability were assessed 48 hpi. Pool of 3 independent experiments. Error bars represent SEM. One-way ANOVA with post-test Dunnett. **(B)** Mouse primary hepatocytes were infected with GFP-expressing *P. berghei* sporozoites and the culture medium was replaced by medium with different concentrations of glucose 2 hpi. Parasite development and the total number of infected cells were assessed at 48 hpi by flow cytometry by determining the fluorescence intensity and the number of GFP<sup>+</sup> cells, respectively. One experiment. One-way ANOVA with post-test Dunnett. Error bars represent SD. ns - not significant, \*  $p < 0.05$ , \*\*  $p < 0.01$  and \*\*\*  $p < 0.001$ .

**Supplementary Figure 2 - Gating strategy to determine 2-NBDG uptake and parasite development by flow cytometry.** Huh7 cells were infected with RFP-expressing *P. berghei* sporozoites and incubated with 2-NBDG-containing medium at different time points of infection. Dot plot represents non-infected and infected (RFP<sup>+</sup>) cells of one replicate from the 48 h time point. Left histogram shows the different fluorescence intensity displayed by non-developing (RFP<sup>low</sup>) and developing (RFP<sup>high</sup>) parasites. Right histogram exemplifies the 2-NBDG uptake by the different cell populations (non-infected, non-developing and developing parasites), assessed as an increased fluorescence intensity in the green channel.

**Supplementary Figure 3 - *P. berghei*-infected Huh7 cells and developing parasites uptake more 2-NBDG than non-infected and naïve cells.** Huh7 cells were infected with RFP-expressing *P. berghei* sporozoites and incubated for 30 min with 2-NBDG-containing medium at 48 hpi. **(A)** Representative images of *P. berghei*-infected Huh7 cells with or without incubation with 2-NBDG. A non-infected cell, an infected cell, and a parasite are outlined in yellow, pink and red, respectively. Scale bar, 20  $\mu\text{m}$ . **(B)** Quantification of 2-NBDG uptake by naïve (grey bar), non-infected cells (yellow bar), infected cells (pink bar) and developing parasites (red bar), as assessed by live fluorescence microscopy.

Representative experiment out of 2 independent experiments. One-way ANOVA with post-test Bonferroni and two-tailed Mann-Whitney test, respectively. Error bars represent SD. \*\*\*  
 $p < 0.001$

**Supplementary Figure 4 - Quantification of the percentage of MHV-68-YFP-infected cells by flow cytometry.** Percentage of YFP<sup>+</sup> cells 24 hpi with different loads of MHV-68-YFP.

**Supplementary Figure 5 – 2-NBDG uptake by non-infected Huh7 cells with GLUT1 knockdown.** 2-NBDG uptake by non-infected cells of the different cell lines with GLUT1 knockdown at 48 hpi, assessed by flow cytometry. Pool of 4 independent experiments. Error bars represent SEM. One-way ANOVA with post-test Dunnett. ns - not significant and \*\*\*  $p < 0.001$ .

**Supplementary Figure 6 - Chemical inhibition of GLUT1-mediated glucose transport by WZB117 affects *P. berghei* hepatic infection ex vivo. (A)** Mouse primary hepatocytes were infected with luciferase-expressing *P. berghei* sporozoites and 2 h later the culture medium was replaced by medium containing WZB117. Parasite load (luminescence) and cell viability were assessed at 48 hpi. Pool of 2 independent experiments. Error bars represent SEM. One-way ANOVA with post-test Dunnett. **(B)** Mouse primary hepatocytes were infected with GFP-expressing *P. berghei* sporozoites and 2 h later the culture medium was replaced by medium containing WZB117. Parasite development was assessed by flow cytometry at 48 hpi. Error bars represent SD. One-way ANOVA with post-test Dunnett. ns - not significant, \*  $p < 0.05$ , \*\*  $p < 0.01$  and \*\*\*  $p < 0.001$ .

**Supplementary Figure 7 - GLUT1 expression is not altered in *P. berghei*-infected cells.** qPCR quantification of GLUT1 transcriptional expression in FACS-sorted infected and non-infected Huh7 cells. Each time point represents a pool of 2 independent sorting experiments. Error bars represent SD. Two-tailed Mann-Whitney test. ns – not significant.



**Supplementary Figure 8 - Increased GLUT1-dependent glucose uptake into *P. berghei*-infected cells is independent of AMPK. (A)** Knockdown efficiency upon transfection in Huh7 cells of the siRNAs used against the  $\alpha 1$  and  $\alpha 2$  subunits of AMPK. **(B)** Huh7 cells with the knockdown of  $\alpha 1$  or/and  $\alpha 2$  subunits of AMPK were infected with GFP-expressing *P. berghei* sporozoites and parasite development was assessed by flow cytometry at 48 hpi. Pool of 2 independent experiments. Error bars represent SD. One-way ANOVA with post-test Dunnett. **(C)** 2-NBDG uptake at 48 hpi by developing parasites-containing cells with the knockdown of  $\alpha 1$  or/and  $\alpha 2$  subunits of AMPK, assessed by flow cytometry. Representative experiment out of 2 independent experiments. Error bars represent SD. One-way ANOVA with post-test Dunnett. ns - not significant, \*\*\*  $p < 0.001$ .

**Supplementary Figure 9 - PI3K stimulation or inhibition does not impact *P. berghei* infection of Huh7 cells neither does it alter glucose uptake by infected cells.** Huh7 cells were infected with luciferase-expressing *P. berghei* sporozoites and 2 h later were treated with different concentrations of **(A)** insulin or **(B)** Wortmannin. Parasite load (luminescence) was assessed after 48 h. Representative experiment out of 2 and pool of 3 independent experiments, respectively. Error bars represent SD. To evaluate possible effects on glucose uptake, Huh7 cells were infected with RFP-expressing *P. berghei* sporozoites and the treatment with different concentrations of insulin **(C)** and Wortmannin **(D)** was initiated 2 hpi. 2-NBDG uptake by developing parasites-containing cells was assessed at 48 hpi by flow cytometry. Pool of 2 independent experiments for both insulin and Wortmannin. Error bars represent SD. All panels: one-way ANOVA with post-test Dunnett. ns - not significant.

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