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**AMA1 and MAEBL are important for *Plasmodium falciparum* sporozoite
infection of the liver**

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Summary

The malaria sporozoite injected by a mosquito migrates to the liver by traversing host cells. The sporozoite also traverses hepatocytes before invading a terminal hepatocyte and developing into exoerythrocytic forms. Hepatocyte infection is critical for parasite development into merozoites that infect erythrocytes, and the sporozoite is thus an important target for antimalarial intervention. Here, we investigated two abundant sporozoite proteins of the most virulent malaria parasite *Plasmodium falciparum* and show that they play important roles during cell traversal and invasion of human hepatocytes. Incubation of *P. falciparum* sporozoites with R1 peptide, an inhibitor of AMA1 that blocks merozoite invasion of erythrocytes, strongly reduced cell traversal activity. Consistent with its inhibitory effect on merozoites, R1 peptide also reduced sporozoite entry into human hepatocytes. The strong but incomplete inhibition prompted us to study the AMA-like protein, MAEBL. MAEBL-deficient *P. falciparum* sporozoites were severely attenuated for cell traversal activity and hepatocyte entry *in vitro* and for liver infection in humanized chimeric liver mice. This study shows that AMA1 and MAEBL are important for *P. falciparum* sporozoites to perform typical functions necessary for infection of human hepatocytes. These two proteins therefore have important roles during infection at distinct points in the lifecycle, including the blood, mosquito and liver stages.

Keywords: Malaria, sporozoite, cell traversal, invasion, hepatocyte, humanized mice

Running title: AMA1 and MAEBL in *P. falciparum* sporozoites

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Background

Malaria is transmitted in 95 countries and territories and approximately 2 billion people are at risk from being infected. This disease is caused by infection with *Plasmodium* parasites that, in 2015, were responsible for over 220 million clinical infections and approximately 630,000 deaths (WHO, 2015, Gething *et al.*, 2016). Of the six *Plasmodium* spp. that infect humans, *P.*

falciparum is responsible for the majority of deaths, with most casualties involving pregnant women and young children living in sub-Saharan Africa (WHO, 2015, Gething *et al.*, 2016).

Infection by malaria parasites begins with the injection of sporozoites into the skin of the human host by an infectious *Anopheles* mosquito. Sporozoites are transported to the liver by the blood and infect hepatocytes, each developing into thousands of merozoites per hepatocyte. Once differentiation into merozoites is completed within the hepatocyte, the parasites egress into the bloodstream where they infect erythrocytes, initiating the symptomatic phase of malaria.

Rodent models of malaria have been used to develop an important understanding of how sporozoites transmigrate through mammalian cells as they navigate from the skin to the liver and infect hepatocytes (reviewed by (Menard *et al.*, 2013)). During this journey, the sporozoite encounters a variety of host cells and cellular barriers which it overcomes using the remarkable process of cell traversal. The ability to actively move through cells was first described during the study of sporozoite-macrophage interactions (Vanderberg *et al.*, 1990). Further research has determined that traversal involves dermal fibroblasts and lymphocytes (Amino *et al.*, 2006, Coppi *et al.*, 2007, Amino *et al.*, 2008), Kupffer cells (Pradel *et al.*, 2001, Ishino *et al.*, 2004,

Frevert *et al.*, 2005, Tavares *et al.*, 2013), sinusoidal endothelial cells (Tavares *et al.*, 2013) and hepatocytes (Mota *et al.*, 2001, Frevert *et al.*, 2005).

Far less is known about cell traversal by malaria sporozoites that cause disease in humans. Important differences have been reported between different *Plasmodium* spp., highlighting the need to study human malaria parasites where possible (Silvie *et al.*, 2006, Kaushansky *et al.*, 2011).

Although *P. falciparum* sporozoites traverse human macrophages and hepatocytes *in vitro* (van Schaijk *et al.*, 2008, Behet *et al.*, 2014, Dumoulin *et al.*, 2015, Yang *et al.*, 2017), there is a paucity of knowledge surrounding the molecular basis for these events (reviewed by (Yang *et al.*, 2016)). Recently it was shown that GAPDH on the sporozoite surface interacts with CD68 on Kupffer cells to facilitate traversal through the macrophages (Cha *et al.*, 2016) and that SPECT and PLP1 are essential for *P. falciparum* traversal of hepatocytes (Yang *et al.*, 2017).

It is currently unclear why sporozoites traverse hepatocytes before productively infecting them. However, the concentration of heparan sulfate proteoglycans (HSPGs) on the host cell surface is a key determinant that signals sporozoite to switch from traversal to invasion and the parasite's decision to change takes time (Coppi *et al.*, 2007).

Invasion of hepatocytes by *P. falciparum*, *P. berghei* and *P. yoelii* sporozoites involves the conserved 6-Cys proteins, P36 and P52. Genetic disruption of the genes encoding them results in sporozoites that either cannot enter hepatocytes (Ishino *et al.*, 2005) or in parasites that developmentally arrest soon after entry as they do not produce a parasitophorous vacuole

(Labaied *et al.*, 2007, VanBuskirk *et al.*, 2009). Recently, P36 was shown to bind the hepatocyte surface protein Ephrin A2 receptor to facilitate the formation of the parasitophorous vacuole (Kaushansky *et al.*, 2015). However, it is believed that other sporozoite proteins also contribute to hepatocyte invasion.

In this study, we have investigated the role of two conserved parasite proteins, apical merozoite antigen 1 (AMA1) and merozoite apical erythrocyte expressed ligand (MAEBL), both of which play important roles at different stages of the *Plasmodium* lifecycle. AMA1 is a merozoite protein stored in micronemes and secreted to the parasite surface (Healer *et al.*, 2002) where it plays an important role in erythrocyte invasion (Thomas *et al.*, 1984, Anders *et al.*, 1998, Triglia *et al.*, 2000). AMA1 cannot be genetically disrupted and is essential in blood stages of different *Plasmodium* species (Triglia *et al.*, 2000, Giovannini *et al.*, 2011, Bargieri *et al.*, 2013). In *P. falciparum*, AMA1 can be blocked by inhibitory peptides such as R1 (Harris *et al.*, 2005, Harris *et al.*, 2009). This peptide binds the AMA1 hydrophobic pocket (Richard *et al.*, 2010) and prevents RON2 binding (Tonkin *et al.*, 2011). R1-treated merozoites can attach strongly to erythrocytes and deform them but cannot invade the cells (Harris *et al.*, 2005, Richard *et al.*, 2010, Riglar *et al.*, 2011). This suggests that AMA1 is involved in invasion after initial attachment and reorientation, likely in establishing the tight junction (Srinivasan *et al.*, 2011). The AMA-RON interaction has been shown to be an attractive target for therapeutic intervention (Srinivasan *et al.*, 2014).

AMA1 is abundantly expressed in sporozoites (Silvie *et al.*, 2004, Giovannini *et al.*, 2011, Lindner *et al.*, 2013). Whilst conditional disruption of AMA1 in the rodent malaria parasite *P. berghei* had no effect on cell traversal activity or hepatocyte invasion (Giovannini *et al.*, 2011), anti-AMA1 monoclonal antibodies strongly reduced hepatocyte invasion by *P. falciparum* sporozoites (Silvie *et al.*, 2004). The reason for this discrepancy has not been resolved experimentally but, in addition to different host tropism, may involve functional redundancy by other parasite proteins (reviewed in (Harvey *et al.*, 2014, Yang *et al.*, 2016)).

MAEBL is a chimeric protein with an AMA-like N-terminus and a C-terminus similar to erythrocyte binding antigen 175 (EBA175) (Kappe *et al.*, 1998). MAEBL is conserved in *Plasmodium* spp. and clusters phylogenetically with AMA4 of *T. gondii* (Parker *et al.*, 2016). Whilst MAEBL can bind erythrocytes (Kappe *et al.*, 1998), genetic disruption had no effect on blood-stage growth, indicating it is not solely essential for erythrocyte invasion (Kariu *et al.*, 2002, Fu *et al.*, 2005, Saenz *et al.*, 2008). In contrast, MAEBL-deficient sporozoites cannot invade mosquito salivary glands (Kariu *et al.*, 2002, Saenz *et al.*, 2008). Two isoforms of MAEBL are expressed in sporozoites as a result of alternate splicing of the 3' exons (Singh *et al.*, 2004) and the transmembrane isoform is essential for *P. falciparum* sporozoites to invade salivary glands (Saenz *et al.*, 2008). The fact that MAEBL is also synthesized by salivary gland sporozoites suggests it may have a function in sporozoites during interactions with the mammalian host. In support of this hypothesis, antibodies specific to both MAEBL isoforms strongly impair *P. falciparum* sporozoite invasion of hepatocytes (Peng *et al.*, 2016) and

antibodies against *P. yoelii* MAEBL also reduce hepatocyte entry (Preiser *et al.*, 2004).

However, direct genetic evidence for such a role has not been reported.

Here, we use an inhibitor combined with molecular genetics to investigate the function of AMA1 and MAEBL in *P. falciparum* sporozoites. Our data indicates that both proteins play important functions in *P. falciparum* during infection of human hepatocytes.

Results

R1 peptide reduces *P. falciparum* cell traversal activity and hepatocyte entry. AMA1 is a micronemal protein in merozoites (Healer *et al.*, 2002) and sporozoites (Silvie *et al.*, 2004).

Since the *PfAMA1* gene cannot be directly disrupted (Triglia *et al.*, 2000), we studied the role of this protein in sporozoites using R1 peptide (Harris *et al.*, 2005). To investigate cell traversal, salivary gland sporozoites of the strain NF54, the parent of 3D7, were incubated with HC-04 hepatocytes in the presence of FITC-dextran and increasing concentrations of R1 peptide. We observed that cell traversal activity by sporozoites was significantly reduced in the presence of R1 peptide in a dose-dependent manner, ranging from $9.0 \pm 0.9\%$ inhibition at 0.1 mg/ml to a maximum of $61.0 \pm 3.0\%$ inhibition at 1.0 mg/ml ($P < 0.0290$) (Fig. 1A). This corresponded to 50% inhibition of cell traversal using 0.7 mg/ml. In contrast, addition of R1 scrambled peptide control had no significant effect on cell traversal (Fig. 1A). This demonstrates that R1 peptide significantly reduces cell traversal activity by *P. falciparum* NF54 sporozoites.

The primary effect of R1 peptide on merozoites is inhibition of invasion into erythrocytes, so we tested whether it has such an effect on sporozoite invasion into hepatocytes. The number of intracellular and extracellular sporozoites following treatment with R1 peptide or R1 scrambled peptide was quantified by immunofluorescence microscopy of differentially labeled parasites (Renia *et al.*, 1988). The number of sporozoites inside hepatocytes was reduced by $59 \pm 0.1\%$ by R1 peptide at 1.0 mg/ml relative to DMSO ($P=0.0025$) whilst R1 scrambled peptide did not significantly affect entry ($P=0.0732$) (Fig. 1B). The reduction in hepatocyte entry represents a similar degree of inhibition to that observed for cell traversal (Fig. 1). To investigate whether R1 peptide affects sporozoite motility, we performed gliding motility assays with salivary gland sporozoites (Stewart *et al.*, 1988). This revealed no significant inhibition of gliding motility by R1 peptide ($P=0.9993$) (Fig. 1C). Therefore, R1 peptide reduces cell traversal and invasion into hepatocytes, consistent with AMA1 playing an important role during these processes.

In merozoites, R1 peptide prevents AMA1 binding to the RON complex by outcompeting the RON2 binding site (Tonkin *et al.*, 2011). Some members of the RON complex are also expressed in sporozoites (Tufet-Bayona *et al.*, 2009, Lindner *et al.*, 2013, Risco-Castillo *et al.*, 2014). To examine expression and subcellular localization of the RON complex in *P. falciparum* sporozoites, we performed immunofluorescence microscopy using antibodies to RON4, an important member of the merozoite RON complex (Riglar *et al.*, 2011). RON4 expression was detected at the apex of sporozoites in two puncta (Fig. 1D) consistent with the rhoptries observed

in RON4-labelled merozoites (Riglar *et al.*, 2011). Several sporozoites showed RON4 immediately extracellular to the parasite, suggesting it was secreted as the parasite was interacting with the host cell (Fig. 1D) as is observed with merozoites (Riglar *et al.*, 2011). Thus, an important member of the merozoite RON complex that is involved in erythrocyte invasion is expressed by *P. falciparum* sporozoites and secreted from rhoptries when the parasite is in close association with hepatocytes.

Taken together, these experiments show that RON4 is expressed in *P. falciparum* sporozoites and that an inhibitory AMA-binding peptide reduces sporozoite cell traversal activity and invasion of hepatocytes, suggesting that AMA1 plays important role in infectious sporozoites.

Generation of *P. falciparum* parasites lacking the AMA-like protein MAEBL. In

Toxoplasma, four AMA1 and three RON2 paralogs can mediate host cell invasion. It is currently not known if *Plasmodium* spp express multiple AMA-RON proteins. However, the incomplete inhibition of cell traversal and hepatocyte invasion by R1 peptide raised the possibility that another protein may facilitate these functions in sporozoites and our attention was directed to the AMA-like protein MAEBL. *Plasmodium* sporozoites abundantly express both AMA1 (Silvie *et al.*, 2004) and MAEBL (Kappe *et al.*, 1998). The M1 and M2 domains of MAEBL share similarity with AMA1 in *P. falciparum* (Blair *et al.*, 2002) and we determined this to be 29.8% similarity using *P. falciparum* strain 3D7 genome sequence data (Supp. Fig. 1).

To investigate the possible role of MAEBL in sporozoite-hepatocyte interactions, we generated *P. falciparum* NF54 parasites in which the *MAEBL* gene was disrupted by double-crossover homologous recombination so that neither the transmembrane or soluble isoform would be expressed (Fig. 2A). Two knockout clones with the expected genotype, F11 and B9, were identified by Southern blot analysis (Fig. 2B). Using immunofluorescence microscopy with antibodies raised to the cytoplasmic tail of MAEBL, we observed expression in sporozoites that partly co-localized with the micronemal protein AMA1 in sporozoites extracted from mosquito hemolymph (Fig. 2C), confirming MAEBL was localized in micronemes as shown previously in *P. berghei* (Kariu *et al.*, 2002). In Δ *MAEBL* parasites, no signal was detected with anti-MAEBL antibodies whilst AMA1 expression remained detectable (Fig. 2C). Thus, *P. falciparum* hemolymph-derived sporozoites express the full-length isoform of MAEBL and disruption of the *MAEBL* locus resulted in loss of protein expression in sporozoites.

The asexual intraerythrocytic growth rate of each *MAEBL* knockout clone was evaluated. No difference relative to NF54 was observed (Fig. 3A) as shown previously in *P. berghei* and *P. falciparum* (Fu *et al.*, 2005, Saenz *et al.*, 2008). Parasites were differentiated to gametocytes and fed to *Anopheles stephensi* mosquitoes. No difference in the number of mature gametocytes (Fig. 3B), mosquito midgut oocysts (Fig. 3C) or hemolymph sporozoites (Fig. 3D) was observed compared to NF54, consistent with previous *P. falciparum* data (Saenz *et al.*, 2008). However, a severe defect was apparent when salivary gland sporozoites were quantified ($P=0.0003$) (Fig. 3E). These results agree with previous reports that MAEBL is essential for invasion of mosquito

salivary glands by *P. berghei* and *P. falciparum* sporozoites (Kariu *et al.*, 2002, Saenz *et al.*, 2008).

Collectively, these results confirm that the *PfMAEBL* locus was successfully disrupted, resulting in mutant sporozoites that behave as reported previously.

MAEBL is required for *P. falciparum* sporozoite traversal of human hepatocytes. A role for MAEBL in cell traversal activity has not been reported previously. Since $\Delta MAEBL$ sporozoites do not invade salivary glands, we isolated sporozoites from the hemolymph for subsequent studies. To establish the experimental conditions, the number of hemolymph sporozoites egressing from oocysts was quantified over consecutive days. NF54 and $\Delta MAEBL$ sporozoites started to egress into the hemolymph from day 11 post-bloodmeal and this increased in equal numbers between strains until day 15 (Fig. 4A). However, on day 16 post-bloodmeal, $\Delta MAEBL$ sporozoite numbers increased significantly in hemolymph and remained higher than NF54 until day 18 (Fig. 4A), presumably due to their inability to invade salivary glands like their NF54 parent (Fig. 4A and 3E). To determine whether hemolymph sporozoites were cell traversal competent, sporozoites were extracted from mosquitoes on days 12, 14 and 16 post-bloodmeal and incubated with HC-04 cells in the presence of FITC-dextran, as described above. After 2.5 hours of incubation, approximately 15% of the hepatocytes became dextran-positive as a result of cell traversal irrespective of the day of hemolymph extraction (Fig. 4B). In contrast, $\Delta MAEBL$ sporozoites caused minimal uptake of dextran into the cells, with the levels being consistently

low on different days (Fig. 4B). Quantification of traversal using both $\Delta MAEBL$ clones on day 14 post-bloodmeal revealed an 82% reduction compared to NF54 ($P < 0.001$), which was similar to the background levels obtained with media alone (87% less dextran uptake than NF54) (Fig. 4C). This shows that *P. falciparum* sporozoites isolated from hemolymph do not differ significantly in their cell traversal capability on different days of extraction and, importantly, that MAEBL is required for traversal of hepatocytes.

To determine whether disruption of *PfMAEBL* affected gliding motility, CSP-positive trails on coverslips were quantified using hemolymph-derived sporozoites. The parasites were actively motile and no significant difference was observed between NF54 and $\Delta MAEBL$ sporozoites (Fig. 4D). Taken together, these results demonstrate that MAEBL is required for cell traversal activity but not gliding motility by *P. falciparum* sporozoites.

MAEBL plays an important role in *P. falciparum* sporozoite invasion of hepatocytes. To investigate whether MAEBL is involved in sporozoite invasion of hepatocytes, sporozoites were incubated with HC-04 cells for 3 hours or 48 hours before the number of intracellular parasites was quantified by immunofluorescence microscopy using differential surface labeling of intracellular and extracellular parasites. At both time points, the number of intracellular $\Delta MAEBL$ parasites was reduced by approximately 80% compared to NF54 ($P < 0.0160$) (Fig. 5C) demonstrating a severe defect in host cell entry. Interestingly, the proportion of intracellular NF54 parasites at 48 hours was significantly less than at 3 hours (Fig. 5A, B). While this could

be partly attributed to hepatocyte proliferation (3.7-fold increase; $P=0.0043$) (Fig. 5C), it suggested that parasites might also be dying. To allow for direct comparisons of parasite numbers between time points, we analyzed the total number of intracellular parasites per well. This revealed that intracellular parasite numbers decreased significantly between the two time-points (NF54 had 47% reduction; $P=0.0025$), suggesting that a subset of intracellular sporozoites at 3 hours could not develop into exoerythrocytic forms (EEFs), as expected. Importantly, the number of $\Delta MAEBL$ parasites relative to NF54 parasites was significantly less at both time points (82-89% reduction at 3 hours, $P<0.0026$ and 73-78% reduction at 48 hours, $P=0.0079$) (Fig. 5D). Collectively, this shows that *P. falciparum* sporozoites lacking MAEBL are severely impaired for hepatocyte invasion, consistent with an important role for this protein in infection of hepatocytes.

To assess the function of MAEBL during hepatocyte infection *in vivo*, homozygous urokinase plasminogen activator-severe combined immunodeficiency mice (uPA^{+/+}-SCID) transplanted with primary human hepatocytes to regenerate chimeric human livers (Mercer *et al.*, 2001) were co-infected intravenously with NF54 and $\Delta MAEBL$ sporozoites isolated from hemolymph. Whilst all mice had approximately equal human chimerism, co-infection of the same mice was employed as a strategy to reduce potential differences in infectivity between mice. The parasites were provided a further 6 days to differentiate into exoerythrocytic forms and amplify their nuclear DNA before parasite liver load was measured in each mouse by quantitative PCR (qPCR). We followed the same protocol published previously, which reported

the detection of 1 infected hepatocyte in 25% of whole liver (Foquet *et al.*, 2013). Using oligonucleotides specific for NF54 and $\Delta MAEBL$ genomes, we observed that NF54 sporozoites infected the livers of humanized mice and developed into exoerythrocytic forms; however, a >600-fold reduction in $\Delta MAEBL$ parasites was observed in the same livers (Fig. 5E). Taken together, these experiments validate that MAEBL is important for *P. falciparum* infection of human hepatocytes *in vivo*.

Discussion

The liver stage of *Plasmodium* infection precedes blood stage malaria and thus represents an important point to study parasite biology with the hope of informing future development of antimalarial interventions. Whilst rodent models of malaria have provided a large body of knowledge, it is important to study human malaria parasites where possible because important differences in biology occur between *Plasmodium* spp. For example, *P. berghei* and *P. yoelii* sporozoites develop into exoerythrocytic forms within many mouse and human cell types, and can use different invasion pathways to enter the cells (Silvie *et al.*, 2006, Silvie *et al.*, 2007); however, *P. falciparum* sporozoites only productively infect human hepatocytes (Silvie *et al.*, 2006). Moreover, expression of CD81 in human hepatocytes is sufficient to allow *P. yoelii* sporozoites to invade but is not sufficient for *P. falciparum* invasion (Silvie *et al.*, 2006). Another example is the activation of the host receptor tyrosine kinase MET by *P. berghei* sporozoites during cell traversal to promote the development of exoerythrocytic forms (EEFs)

(Carrolo *et al.*, 2003) and inhibit hepatocyte apoptosis (Leiriao *et al.*, 2005). This does not occur during cell traversal by *P. falciparum* or *P. yoelii* sporozoites (Kaushansky *et al.*, 2011).

Therefore, our approach in this study was to use the human malaria parasite *P. falciparum* to investigate the function of AMA1 and MAEBL in sporozoites. This revealed important roles for both proteins during cell traversal and during parasite invasion of hepatocytes.

R1 peptide is a specific inhibitor of AMA1 in *P. falciparum* 3D7 merozoites that prevents erythrocyte invasion. This inhibitor caused a strong reduction in cell traversal activity and invasion of human hepatocytes by NF54 sporozoites, suggesting that AMA1 is important for both processes. The concentration required to inhibit cell traversal by 50% was higher than that reported previously for merozoites (Harris *et al.*, 2005). This may reflect variation in the multiplicity of infection between merozoite invasion assays with 3D7 (0.05:1) and sporozoite traversal assays with NF54 (1:1) or it may indicate that AMA1 is stoichiometrically more abundant on sporozoites than merozoites. Alternatively, this might be due to R1 binding to a different sporozoite protein or to differences in the protein composition at the tight junction between merozoites and sporozoites. R1 peptide acts by displacing RON2 binding to AMA1 (Srinivasan *et al.*, 2011, Tonkin *et al.*, 2011). While sporozoites express RON2 and other members of the RON complex (Tufet-Bayona *et al.*, 2009, Lindner *et al.*, 2013), it is currently not known if RON2 is the AMA1 binding partner in sporozoites. RON2 (and RON4) is expressed and secreted by *P. yoelii* sporozoites during entry into CD81-positive hepatocytes (Risco-Castillo *et al.*, 2014) and RON4 is essential for *P. berghei* sporozoites to invade

hepatocytes (Giovannini *et al.*, 2011), so it remains a viable possibility that AMA1 binds RON2, which in turn is bound to RON4. Our analysis of the RON complex in *P. falciparum* sporozoites focused on RON4 as we had suitable reagents. We observed RON4 within two punctate spots in sporozoites, consistent with rhoptries, and also witnessed extracellular RON4 in close proximity to sporozoites interacting with hepatocytes. Since *P. falciparum* invasion is not solely CD81-dependent (Silvie *et al.*, 2006), we could not differentiate traversal from invasion at the molecular level, as has been elegantly established for *P. yoelii* (Risco-Castillo *et al.*, 2014, Risco-Castillo *et al.*, 2015). However, our observations provide evidence that at least one member of the RON complex is secreted by *P. falciparum* sporozoites as they interact with hepatocytes, as would be expected during invasion.

The timing of invasion arrest for R1 peptide-treated merozoites occurs after attachment to the host cell, since the parasites still bind to and deform erythrocytes as they try to invade, but they cannot enter and a nascent parasitophorous vacuole is not produced (Richard *et al.*, 2010, Riglar *et al.*, 2011). This suggests that AMA1 plays a key role in establishing the tight junction (Riglar *et al.*, 2011, Srinivasan *et al.*, 2011), a hypothesis strongly supported by additional studies of AMA-RON binding in *Toxoplasma* (Lamarque *et al.*, 2011, Tyler *et al.*, 2011). Another hypothesis is that AMA1 is not required for tight junction but is important for attachment to the host cell and/or reorientation (Giovannini *et al.*, 2011). In our experiments, R1 peptide-treated sporozoites still attached to hepatocytes and coverslips, suggesting that this

peptide does not directly interfere with parasite adherence to HC-04 hepatocytes but affects a downstream step. The precise point will require further detailed investigation.

Recently it was shown that approximately 50% of *P. yoelii* sporozoites traverse hepatocytes using a transient vacuole (Risco-Castillo *et al.*, 2015). While it is not known whether *P. falciparum* sporozoites also produce a transient vacuole, if so, perhaps R1 peptide inhibits their formation. This could explain the residual traversal observed in our study in the presence of R1 peptide, as the sporozoites could still traverse using the alternate pathway of rupturing the host cell membrane upon entry (Mota *et al.*, 2001, Risco-Castillo *et al.*, 2015). The observation of transient vacuole formation during the entry step of traversal raises the possibility that traversal and invasion involve that same, or very similar, entry steps but different egress steps. This idea is consistent with AMA1 and MAEBL playing a role in both processes, as suggested by this study. The development of tools to study *P. falciparum* traversal by high resolution live imaging is needed to further address this question.

The function of AMA1 in sporozoites has been studied previously using genetics approaches that targeted and disrupted either the AMA1 gene or 3' untranslated region in *P. berghei*. These studies showed that AMA1 has an important function in *P. berghei* merozoite attachment to erythrocytes but plays no role in hepatocyte infection (Giovannini *et al.*, 2011, Bargieri *et al.*, 2013). This raises the question of why, if AMA1 is dispensable in sporozoites, is it expressed so abundantly? In *P. falciparum*, anti-AMA1 antibodies strongly reduce sporozoite invasion of human hepatocytes, suggesting that it is important for liver infection by this

particular parasite species (Silvie *et al.*, 2004). One explanation for the discordant results is the inability to generate a pure population of *P. berghei* AMA1-deficient sporozoites (Giovannini *et al.*, 2011, Bargieri *et al.*, 2013). As the authors noted, it could not be excluded that AMA1-deficient parasites were able to exhibit a normal phenotype because of the availability of low amounts of AMA1 in some parasites. Another plausible explanation is functional redundancy of AMA mediated by one or more other proteins (reviewed in (Harvey *et al.*, 2014)). In *Toxoplasma* and other apicomplexans, three additional AMA1 paralogs and a further two RON2 paralogs facilitate invasion (Poukchanski *et al.*, 2013, Lamarque *et al.*, 2014). It is therefore possible that *Plasmodium* spp. also utilize AMA and/or RON paralogs to interact with host cells, particularly because they also interact with a variety of host cells during their lifecycle.

One candidate for an AMA1 paralog in *Plasmodium* spp. is the chimeric protein MAEBL (Kappe *et al.*, 1998). While AMA1 and MAEBL are not identical, they share similarity and are evolutionarily related (Kappe *et al.*, 1998, Parker *et al.*, 2016). MAEBL-deficient *P. berghei* and *P. falciparum* sporozoites show a striking defect during invasion of mosquito salivary glands (Kariu *et al.*, 2002, Saenz *et al.*, 2008), a finding that was also confirmed in our study. Like AMA1, its precise function during invasion of salivary glands is not yet known. In our study, we sought to investigate whether MAEBL is also involved in sporozoite infection of hepatocytes. Rather than mechanically liberate sporozoites from the midgut as reported previously (Kariu *et al.*, 2002), we isolated sporozoites from the mosquito hemolymph following normal egress of sporozoites from oocysts. NF54 hemolymph sporozoites were motile and their cell traversal

activity remained stable over consecutive days. The hemolymph sporozoites also invaded HC-04 hepatocytes. However, genetic loss of MAEBL caused a profound loss of cell traversal activity and hepatocyte invasion into HC-04 cells yet had no effect on gliding motility. This provides direct genetic proof that MAEBL has an important role during human hepatocyte infection as suggested previously through the use of inhibitory antibodies (Preiser *et al.*, 2004, Peng *et al.*, 2016). This was confirmed *in vivo* using humanized mice with chimeric human livers, where MAEBL-deficient *P. falciparum* sporozoites were severely attenuated for liver infection. Although sporozoites are far more infectious for hepatocytes after they reach salivary glands (Ball *et al.*, 1961, Sultan *et al.*, 1997), they do not have to invade these glands to be infectious, and priming can even begin in the hemolymph (Vanderberg, 1975, Sultan *et al.*, 1997, Kariu *et al.*, 2002). Our study of *P. falciparum* NF54 hemolymph sporozoites detected infection of hepatocytes in humanized mice with approximately 10^3 -fold less efficiency than when we used NF54 salivary gland sporozoites (Yang *et al.*, 2017). This difference is similar to that reported previously for *P. berghei* (Sultan *et al.*, 1997). Nonetheless, our current study has revealed a >600-fold reduction in hepatocyte infectivity *in vivo* when MAEBL expression was disrupted, in agreement with our *in vitro* data, and the previous work of others (Preiser *et al.*, 2004, Peng *et al.*, 2016) that MAEBL is important for hepatocyte infection.

The role of MAEBL in liver infectivity of rats has been assessed using *P. berghei* sporozoites, which showed that midgut sporozoites did not require MAEBL for liver infection (Kariu *et al.*, 2002). It is unclear whether that was a *P. berghei*-specific phenotype or the result

of isolating sporozoites from midguts, or due to another reason. However, our body of results with *P. falciparum* hemolymph sporozoites were highly reproducible and statistically significant and our conclusions are based on these data.

HC-04 cells have been a useful model to examine human liver stage biology (Sattabong et al), because it is the only human hepatocellular carcinoma line that supports complete development of *P. falciparum*, albeit at low infection efficiencies. We have investigated sporozoite-hepatocyte interactions using HC-04 cells and humanized mice with the human pathogen *P. falciparum* and shown that AMA1 and MAEBL have important roles during cell traversal activity and infection of liver cells. The significance of both proteins at disparate stages of the parasite's lifecycle suggests that shared components of the attachment/invasion machinery may be utilized by different zoite forms to enter cells of the human and mosquito hosts. It is known that several steps precede merozoite invasion of erythrocytes, such as primary attachment and reorientation of the parasite, followed by irreversible attachment and junction formation before ultimately ending in the encasement of the invaded merozoite with a parasitophorous vacuole membrane (Beeson *et al.*, 2016). It is not yet clear whether sporozoite invasion of hepatocytes also follows a similar pattern. If so, the data shown here do not allow us to pinpoint exactly where AMA1 and MAEBL act in this cascade or even if they are involved in the same step. However, it is possible that AMA1 and MAEBL are part of a group of proteins such as members of the 6-cys family, P52/P36 (Ishino *et al.*, 2005, Labaied *et al.*, 2007, VanBuskirk *et*

al., 2009, Kaushansky *et al.*, 2015), that act together to mediate successful invasion of sporozoites into hepatocytes.

Methods

Parasite maintenance

The asexual stages of *Plasmodium falciparum* strain NF54 were maintained in human type O positive erythrocytes (Melbourne Red Cross) at 4% hematocrit. RPMI-HEPEs media supplemented with 10% heat inactivated human serum (Melbourne Red Cross) were used to maintain the parasites at 37 °C in 94% N, 5% CO₂, 1% O₂. Sexual forms of the parasite were generated for transmission to mosquitoes using the “crash” method as previously described (Saliba *et al.*, 2013).

Transgenic parasites

P. falciparum NF54 was used to generate all transgenic parasites used in this study. To generate the MAEBL knockout construct, 5' and 3' flanks of the locus were cloned into pCC1 using SacII/SpeI (5' flank) and EcoR1/AvrII (3' flank). Primers for amplification of PfMAEBL (PF3D7_1147800) were: forward primer 5' – GAAGAAGAAAGAGACGTACTTTCAG – 3' and reverse primer 5' – CTTTCATGTGTTTCCTATAATTACAC – 3' (5' flank); forward primer 5' – GTTCGATTCCGAAATAAAAGAAGTTG – 3' and reverse primer 5' – CAGGAGTATCAGTACTATTCCTGCTC – 3' (3' flank). Purified plasmid DNA (80 µg) was

transfected into ring stage NF54 and selected using 5 nM WR92210. Lines were cloned by limiting dilution and genotypes assessed by Southern blot using the digoxigenin kit (DIG) from Roche, according to manufacturer's instructions.

Asexual blood stage growth assay

Highly synchronous trophozoite stage parasites were diluted to 0.5% parasitemia at 2% hematocrit and this was confirmed by flow cytometry (FACSCalibur; BD) using ethidium bromide (10 ug/ml; BioRad) (Sleebbs *et al.*, 2014). Final parasitemia was determined 48 hours later by FACS as above. For each line, triplicate samples of 50,000 cells were counted in each of the three independent experiments. Growth was expressed as a percentage of the parasitemia achieved by the NF54 parent.

Mosquito infection and analysis of parasite development

Five- to seven-day-old female *Anopheles stephensi* mosquitoes (obtained from M. Jacobs-Lorena Johns Hopkins University) were provided a bloodmeal containing asynchronous gametocytes, diluted to 0.3-0.6% stage V gametocytemia. Mosquitoes were sugar starved for 16-24 hours before bloodfeeding and another 48 hours post feeding to select the bloodfed females from a mixed mosquito population. Surviving mosquitoes were provided sugar cubes and water (via cotton wick) *ad libitum*. Oocysts were quantified from midguts isolated from cold-anesthetized and ethanol-killed mosquitoes 7-8 days post-bloodmeal and stained with 0.1% mercurochrome in

water. Hemolymph-derived sporozoites were isolated at days 14-16 post-bloodmeal by perfusion of the mosquito thorax using 100 μ l of RPMI HEPES media with a 29G needle, collecting the flow through in 1 ml tubes. Salivary glands were dissected in RPMI HEPES media from mosquitoes on days 15-18 post-bloodmeal and sporozoites were liberated using pestles and filtered through glass wool to obtain sporozoites used in subsequent assays.

Hepatocyte culture

HC-04 hepatocytes (Sattabongkot *et al.*, 2006) were maintained in IMDM, supplemented with 5% heat-inactivated fetal bovine serum (FBS) at 37 °C in 5% CO₂. Cells were split 1:6 every 2-3 days, once they reached ~ 90% confluency.

Cell traversal assay

The traversal activity of sporozoites was measured using the standard cell-wounding assay described in (Dumoulin *et al.*, 2015). Briefly, 5×10^4 HC-04 cells were seeded into each well of a 48 well plate (Corning, Sigma Aldrich) that has been coated with type IV rat-tail collagen. Hepatocytes were incubated with 5×10^4 sporozoites (multiplicity infection of 1:1) in the presence of 1 mg/ml FITC-labelled dextran (10,000 MW, Sigma Aldrich) 12 hours after seeding. After the 2.5 hour incubation at 37 °C, cells were trypsinized to obtain a single cell suspension. For each condition, triplicate samples of 10,000 cells were counted by flow cytometry (FACSCalibur; BD) for each of three independent experiments.

Gliding motility assay

Eight-well chamber slides (Thermo Fisher Scientific 154534) were coated with CSP antibodies (1:1000 dilution in PBS). 20,000 sporozoites were seeded into each well and allowed to glide for 60 minutes at 37 °C in 5% CO₂ in IMDM supplemented with 10% heat-inactivated human serum. Experiments were stopped by removing the supernatant and fixing with 4% paraformaldehyde at 37 °C for 20 minutes. Primary antibody of PfCSP (1:1000 dilution in 3% BSA) was applied followed by goat anti-mouse Alexa 594 at (1:1000 dilution in 3% BSA). Samples were viewed on a Deltavision Elite microscope (Applied Precision) using an Olympus 163x/1.42 PlanApoN objective equipped with a Coolsnap HQ2 CCD camera. Approximately a total of 50 sporozoites were counted for each condition per each of three independent experiments.

Hepatocyte invasion assay

Coverslips coated in rat tail collagen were seeded with 5×10^4 HC-04 cells using DMEM without glucose (Life Technologies, 11966-025), supplemented with 1 mM sodium pyruvate (Life Technologies, 11360-070); 1% FBS (Cellgro, 35-010-CV); 1X Pen/Strep (Corning, 30-001-Cl); 1X MEM non-essential amino acids without L-glutamine (Sigma-Aldrich, M5550); and 1:500 dilution of Lipid Mixture 1, Chemically Defined (Sigma-Aldrich, L0-288). Sporozoites (5×10^4) were added to the cells 12 hours after seeding and incubated for either 3 or 48 hours.

Coverslips were fixed in 4% paraformaldehyde for 20 minutes at room temperature and processed as described (Renia *et al.*, 1988). Briefly, sporozoites were detected by immunofluorescence staining using mouse monoclonal antibodies against PfCSP (1:1000) and goat anti-mouse Alexa 488 secondary antibodies (1:1000). Entire coverslips were tiled at 20x magnification using a Zeiss AxioObserver system. A series of 60 random tiles were counted (corresponding to 20% of the coverslip). This equated to approximately 7,500 (3 hour incubation) and 35,000 (48 hour incubation) HC-04 cells. The percentage of cells with intracellular sporozoites or the number of intracellular parasites per well was calculated from this dataset. For each condition, duplicate samples from three independent experiments were manually counted.

Immunofluorescence microscopy

Salivary gland sporozoites were dissected on days 15-17 post-blood meal and 5×10^4 parasites were added to an equal number of HC-04 cells that had been seeded on to collagen-coated coverslips 12 hours prior. After 1 hour of incubation at 37 °C, coverslips were fixed for 20 minutes at room temperature (RT) with 4% paraformaldehyde and permeabilized in 0.1% Triton X-100 for 5 minutes and the following primary antibodies were applied in 3% bovine serum albumin (BSA; Sigma) in PBS: rabbit anti-RON4 R785 1:250 (Riglar *et al.*, 2011), ActinRed 555 1:500 (Invitrogen), and mouse monoclonal anti-CSP (2A10) 1:2000 (Nardin *et al.*, 1982). Secondary antibodies used were goat anti-mouse Alexa 488 and donkey anti-rabbit Alexa 647

(1:1000, Invitrogen). Images were captured on a Leica SP8 laser scanning confocal microscope with a 60X/1.4 Oil immersion objective (pinhole, 0.6 AU) and HyD's running in photon counting mode and were then deconvolved using Huygens software (Scientific Volume Imaging, Netherlands). Final files were processed and volume rendered using Imaris software. For hemolymph sporozoites, parasites were isolated on day 14-16 post-blood meal by mosquito perfusion and were air-dried on slides. Sporozoites were fixed for 20 minutes at room temperature with 4% paraformaldehyde/PBS and permeabilized with 0.1% Triton X-100 for 5 min. Primary antibodies were diluted in 3% BSA in PBS and used as follows: mouse monoclonal anti-PfAMA1 (2H4) 1:50 (Yap *et al.*, 2014) and rabbit anti-PfMAEBL antibodies (1:50) raised against the C-terminal tail (a gift from Alan Cowman). Secondary antibodies were goat anti-rabbit Alexa 594 and anti-mouse Alexa 488 (both at 1:1000 in 3% BSA/PBS, ThermoFisher Scientific). Micrographs were acquired on a Deltavision Elite microscope (Applied Precision) using an Olympus 100x/1.42 PlanApoN objective equipped with a Coolsnap HQ2 CCD camera as Z stacks. Images were deconvolved and are represented as maximum intensity projections.

Humanized mice production, infection and processing

uPA^{+/+}-SCID mice were housed in a virus- and antigen-free facility supported by the Health Sciences Laboratory Animal Services at the University of Alberta and cared for in accordance with the Canadian Council on Animal Care guidelines. All protocols involving mice were reviewed and approved by the University of Alberta Health Sciences Animal Welfare Committee

and the Walter and Eliza Hall Institute of Medical Research Animal Ethics Committee. uPA+/+-SCID mice at 5-14 days old received 10^6 human hepatocytes (cryopreserved human hepatocytes were obtained from BioreclamationIVT - Baltimore MD) by intrasplenic injection and engraftment was confirmed 8 weeks post-transplantation by analysis of serum human albumin (Mercer et al., 2001). An inoculum of 2.4×10^5 *P. falciparum* NF54 sporozoites and 2.4×10^5 Δ MAEBL F11 sporozoites freshly isolated from mosquito hemolymph were injected by intravenous tail injection into the same three humanized mice. Livers were obtained 6 days post-infection from CO₂-ethanized mice and individual lobes were cut as described (Foquet et al., 2013). Lobes were pooled and emulsified into a single cell suspension and flash frozen in liquid nitrogen for subsequent gDNA extraction.

Measuring exoerythrocytic development in humanized mice

To quantify parasite load in the chimeric livers, gDNA was isolated from the single cell liver suspensions and Taqman probe-based qPCRs were performed as previously described (Alcoser et al., 2011, Foquet et al., 2013, Yang et al., 2017). To specifically differentiate NF54 from Δ MAEBL genomes from the same mouse samples, the following oligonucleotides were used: For NF54 genomes: MAEBL_F (5'-GAATGGCAAAGGAAGGTGA-3') and MAEBL_R (5'-GTGCCCTCCCTTCATAACA-3') which bind internal to the MAEBL deletion in NF54 but do not amplify a product using Δ MAEBL parasites. For Δ MAEBL genomes: hDHFR_F (5'-ACCTAATAGAAATATATCAGGATCC-3') and hDHFR_R (5'-

GTTTAAGATGGCCTGGGTGA-3') which bind in the hDHFR cassette in Δ MAEBL parasites but do not amplify a product using NF54. Human and mouse genomes were quantified using oligonucleotides specific for prostaglandin E receptor 2 (PTGER2) from each species, as described (Alcoser *et al.*, 2011). All probes were labelled 5' with the fluorophore 6-carboxy-fluorescein (FAM) and contain a double-quencher that includes an internal ZENTM quencher and a 3' Iowa Black[®] quencher from IDT. The following probes were used: MAEBL FAM/AAAACAGCA/ZEN/GGTACAAGTCAATCA/3IABkFQ/, hDHFR FAM/TAAACTGCA/ZEN/TCGTCGCTGTG/3IABkFQ, hPTGER2 FAM/TGCTGCTTC/ZEN/TCATTGTCTCG/3IABkFQ, mPTGER2 FAM/CCTGCTGCT/ZEN/TATCGTGGCTG/3IABkFQ. Standard curves were prepared by titration from a defined number of DNA copies for *P. falciparum* NF54, Δ MAEBL, human and mouse controls. PCRs were performed on a Roche LC80 using LightCycler 480 Probe Master (Roche).

Statistics

All statistics were performed using the Mann Whitney nonparametric test in Graphpad Prism 6.

Declarations

Ethics Statement

All experimental protocols involving mice were conducted in strict accordance with the recommendations in the National Statement on Ethical Conduct in Animal Research of the National Health and Medical Research Council and were reviewed and approved by the Walter and Eliza Hall Institute of Medical Research Animal Ethics Committee (AEC2014.030).

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

ASPY, SL, MTO and SME performed experiments, DD and NMK generated humanized mice, JAB designed and interpreted experiments, all authors contributed to writing the manuscript.

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Figure Legends

Figure 1. R1 peptide impairs cell traversal and hepatocyte entry by *P. falciparum*

sporozoites. (A) Cell traversal inhibition by R1 peptide at increasing concentrations (mg/ml). Controls are vehicle alone (DMSO) or R1 scrambled peptide (R1 scr). (B) Percentage of HC-04 cells with intracellular parasites following treatment with DMSO, R1 peptide or R1 scrambled peptide (R1 scr) for 3 hr. Peptides were 1.0 mg/ml. (C) Quantification of CSP trails derived from gliding motility of salivary gland NF54 sporozoites. No significant differences were observed. (D). Localization of RON4 in *P. falciparum* sporozoites incubated with HC-04 hepatocytes for 1

hour. Yellow, phalloidin (host cell actin); blue, DAPI (host and sporozoite nuclei); green, anti-CSP; red, anti-RON4. Scale bars, 5 μ m.

Figure 2. Generation of *P. falciparum* Δ MAEBL parasites. (A) Schematic representation of allelic exchange to genetically disrupt the *PfMAEBL* gene. (B) Southern blots analysis of NF54 and Δ MAEBL clones F11 and B9. Genomic DNA was digested with NdeI/AflIII/SacII or EcoRI/PacI and hybridized with 5' and 3' probes respectively. (C) Immunofluorescence microscopy of hemolymph sporozoites. Top: MAEBL is expressed in NF54 sporozoites (red) and shares similar localization with AMA1 (green). Bottom: No expression of MAEBL could be detected in Δ MAEBL sporozoites but AMA1 was still expressed.

Figure 3. Characterization of Δ MAEBL parasites. (A) The asexual growth rate of NF54 and Δ MAEBL parasites after 48 hours is not statistically different (n.s.). (B) Percentage of stage V gametocytes produced by NF54 and Δ MAEBL parasites 17 days after initiation of gametocytogenesis is not statistically different. (C) The number of mosquito midgut oocysts produced by NF54 or Δ MAEBL parasites on day 7 post-bloodmeal is not statistically different. (D) Number of hemolymph sporozoites per mosquito isolated 14 days post-bloodmeal is not significantly different between NF54 and Δ MAEBL parasites. (E) Number of salivary gland sporozoites per mosquito. The profound difference is significant (***)P=0.0003). Shown are single

representatives of triplicate experiments. All data is the mean and standard error of the mean (SEM).

Figure 4 MAEBL is required for cell traversal activity by *P. falciparum* sporozoites. (A)

Time course of sporozoite egress from oocysts into hemolymph from days 11 to 18 post-bloodmeal. **P=0.0012, ***P<0.0001. (B) Percentage of traversed (FITC-dextran [FITC-dex] positive) HC-04 hepatocytes by Δ MAEBL and NF54 hemolymph sporozoites. *** Δ MAEBL sporozoites traverse significantly less than NF54 (P=0.00016, 0.00018, 0.0002 on days 12, 14 and 16, respectively). (C) Left: Raw FACS data of one representative of three experiments showing the percentage of FITC-dex positive HC-04 cells following incubation with either NF54 or Δ MAEBL hemolymph sporozoites isolated on day 14 post-bloodmeal or with media alone (no sporozoites). Right: quantification of FITC-dex-positive cells from a second representative of three independent experiments (different to left panel). ***P<0.0001. (D) Left: Micrographs of circumsporozoite protein (CSP)-positive trails created by NF54 (left) and Δ MAEBL (right) hemolymph sporozoites. Right: Quantification of the number of circles per sporozoite trail from 150 independent sporozoites (50 sporozoites each in three independent experiments). No statistical difference was observed (n.s.). All data are the mean and standard error of the mean (SEM) from triplicate independent experiments.

Figure 5 MAEBL is important for invasion of human hepatocytes. (A) Percentage of HC-04 cells with intracellular parasites three hours after incubation with NF54 or Δ MAEBL hemolymph sporozoites. ***P<0.0001. (B) Percentage of HC-04 cells with exoerythrocytic forms 48 hours post incubation with either NF54 or Δ MAEBL parasites. **P=0.0079. (C) Mean number of hepatocytes counted per tile showing that equal numbers were counted at both time points. The increase at 48 hours corresponds to HC-04 proliferation. (D) Total number of intracellular parasites per well after 3 hours and 48 hours of incubation with either NF54 or Δ MAEBL sporozoites. **P<0.0079. All data are the mean and standard error of the mean (SEM) from duplicate independent experiments. (E) Quantification of parasite liver load by real-time PCR of three mice with humanized livers co-infected with NF54 or Δ MAEBL sporozoites. Livers were harvested on day 6 post-infection, when parasite nuclei had replicated exponentially. Values were normalized to a series of pre-tested standards and are mean \pm SD. Human chimerism in each mouse was determined to be (29%, 26% and 25%) by qPCR using oligonucleotides specific to human and mouse PTGER2.

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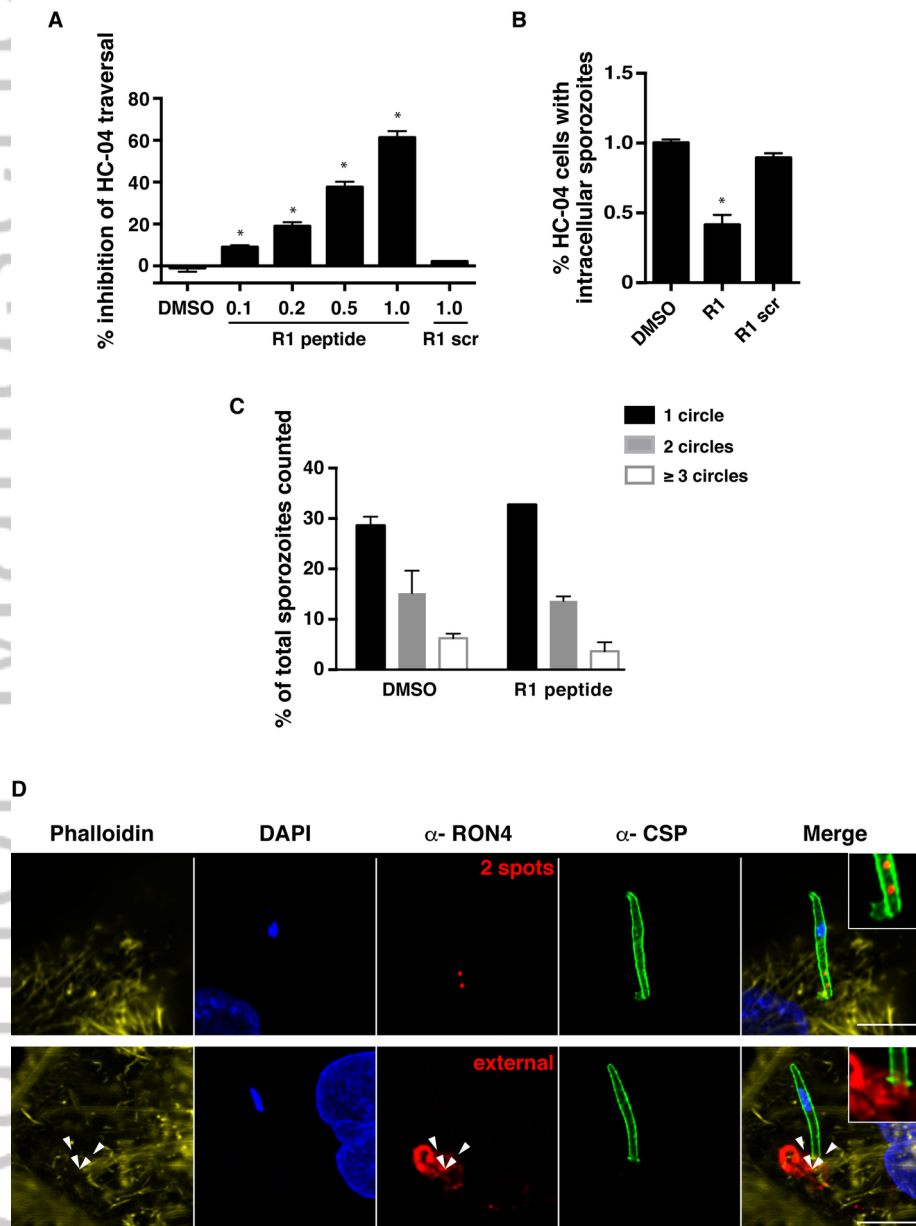
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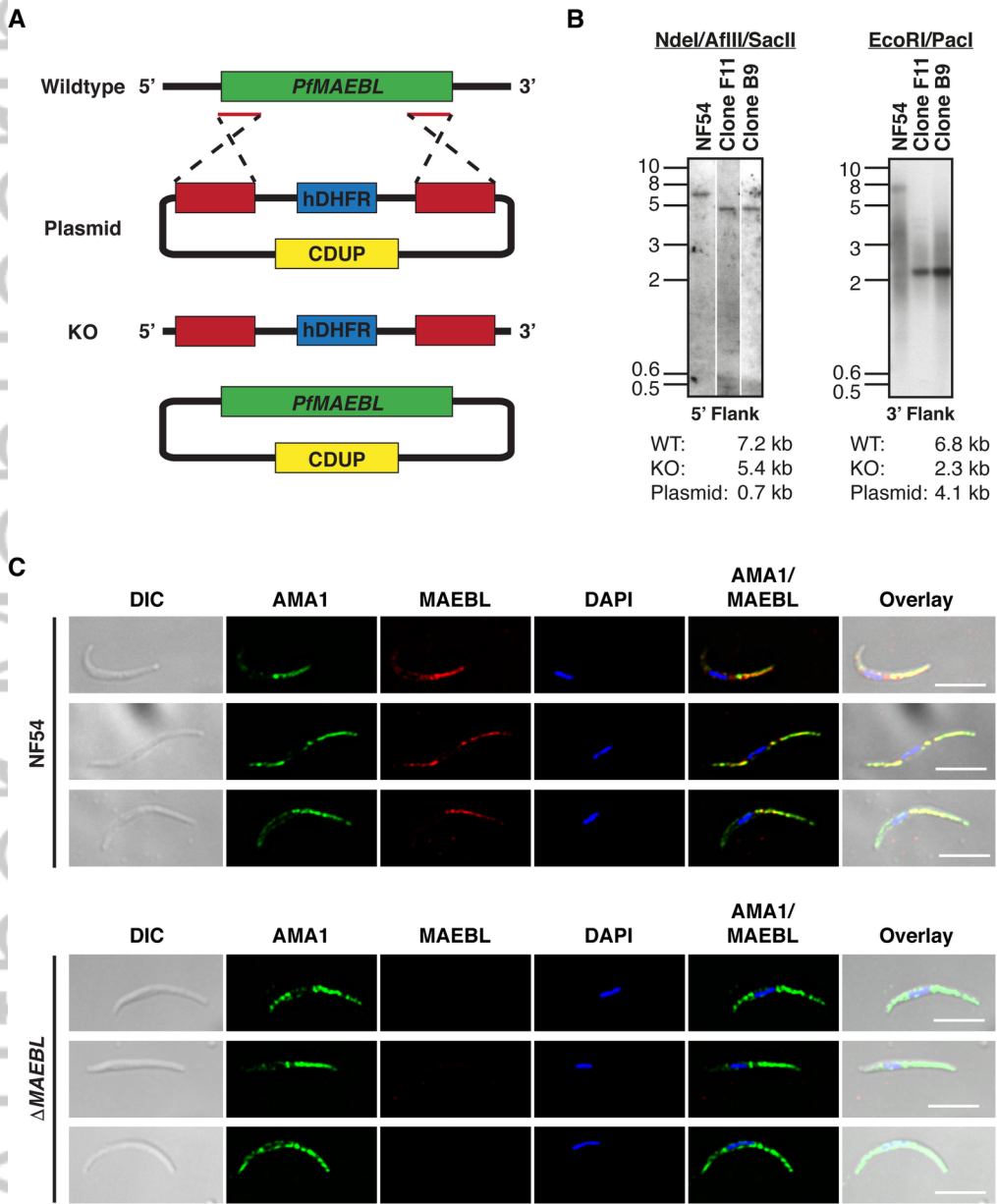
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Figure 1



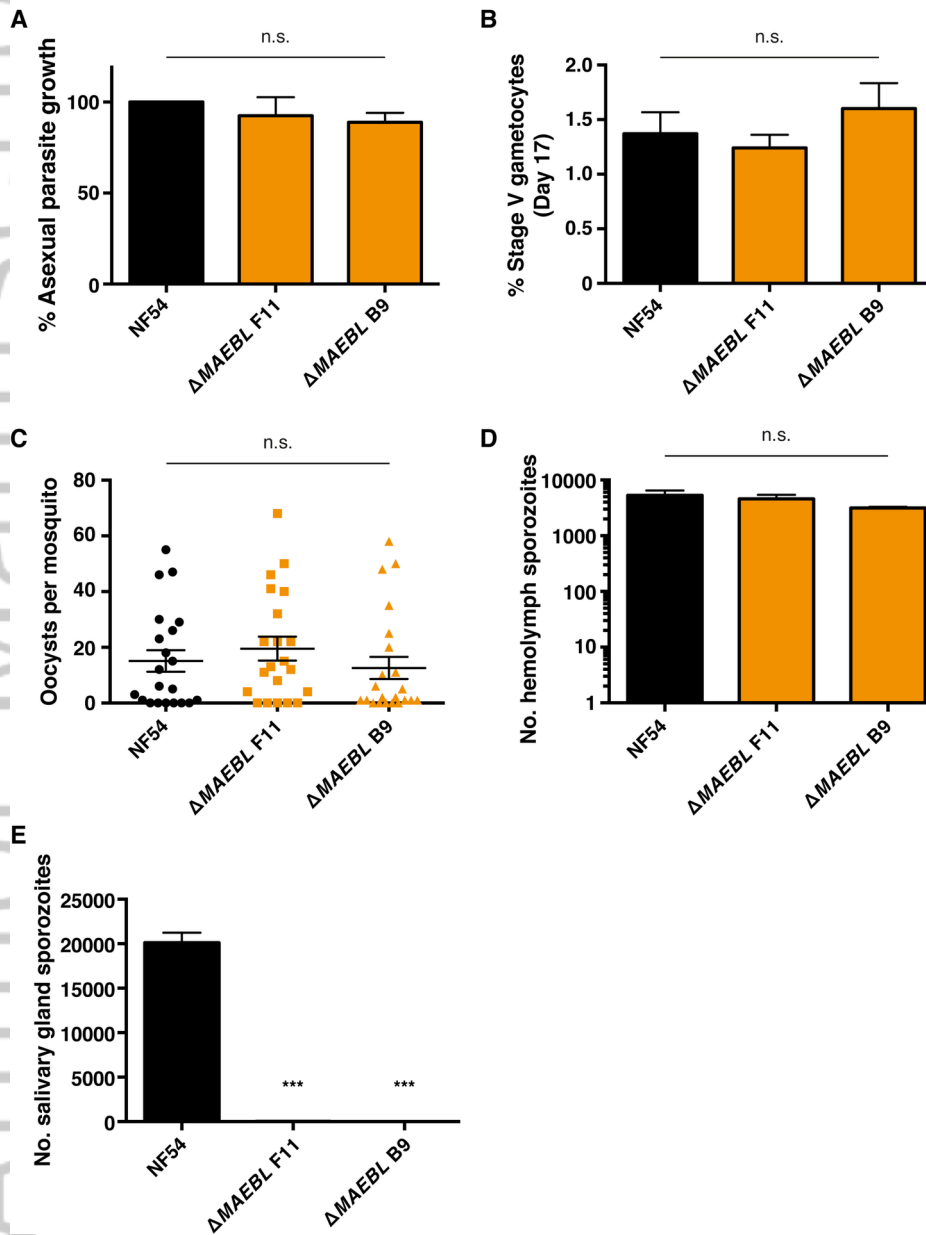
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Figure 2



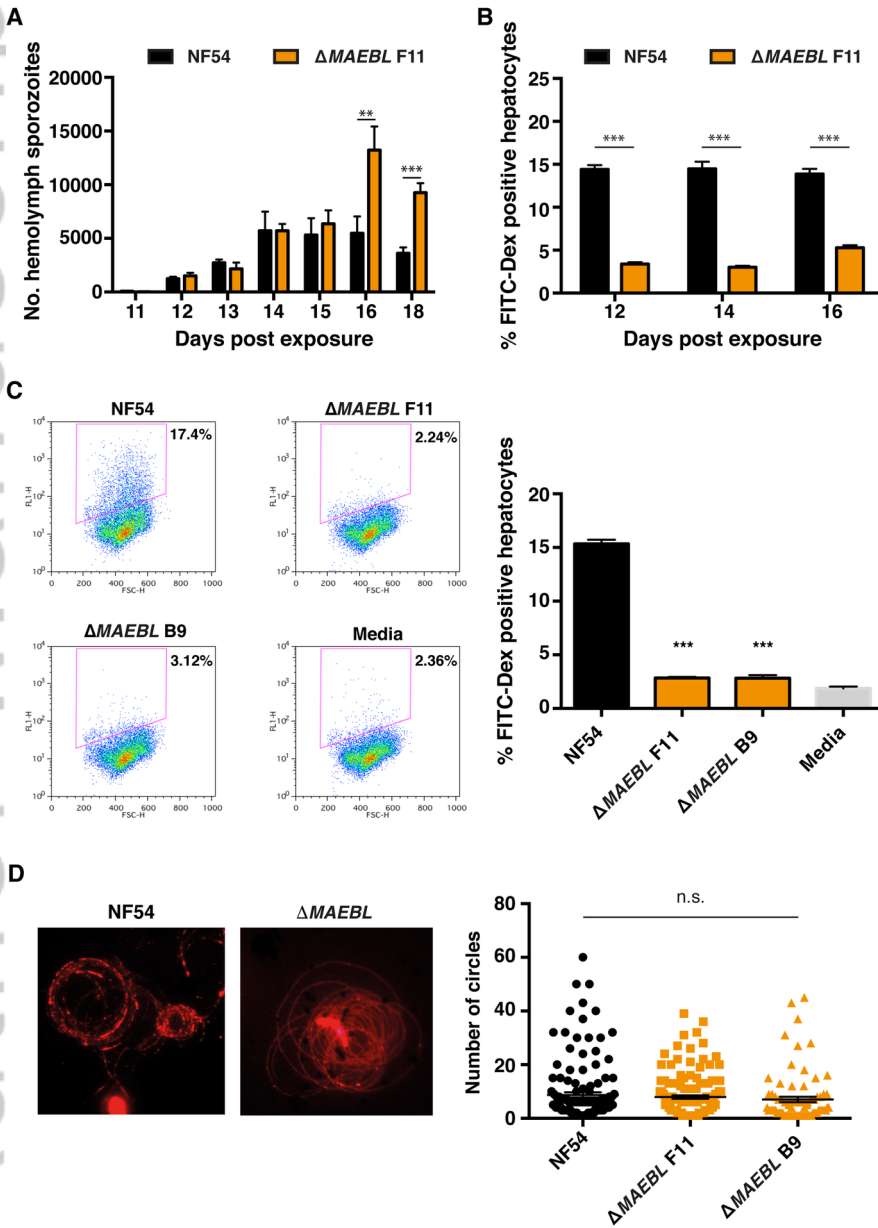
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Figure 3



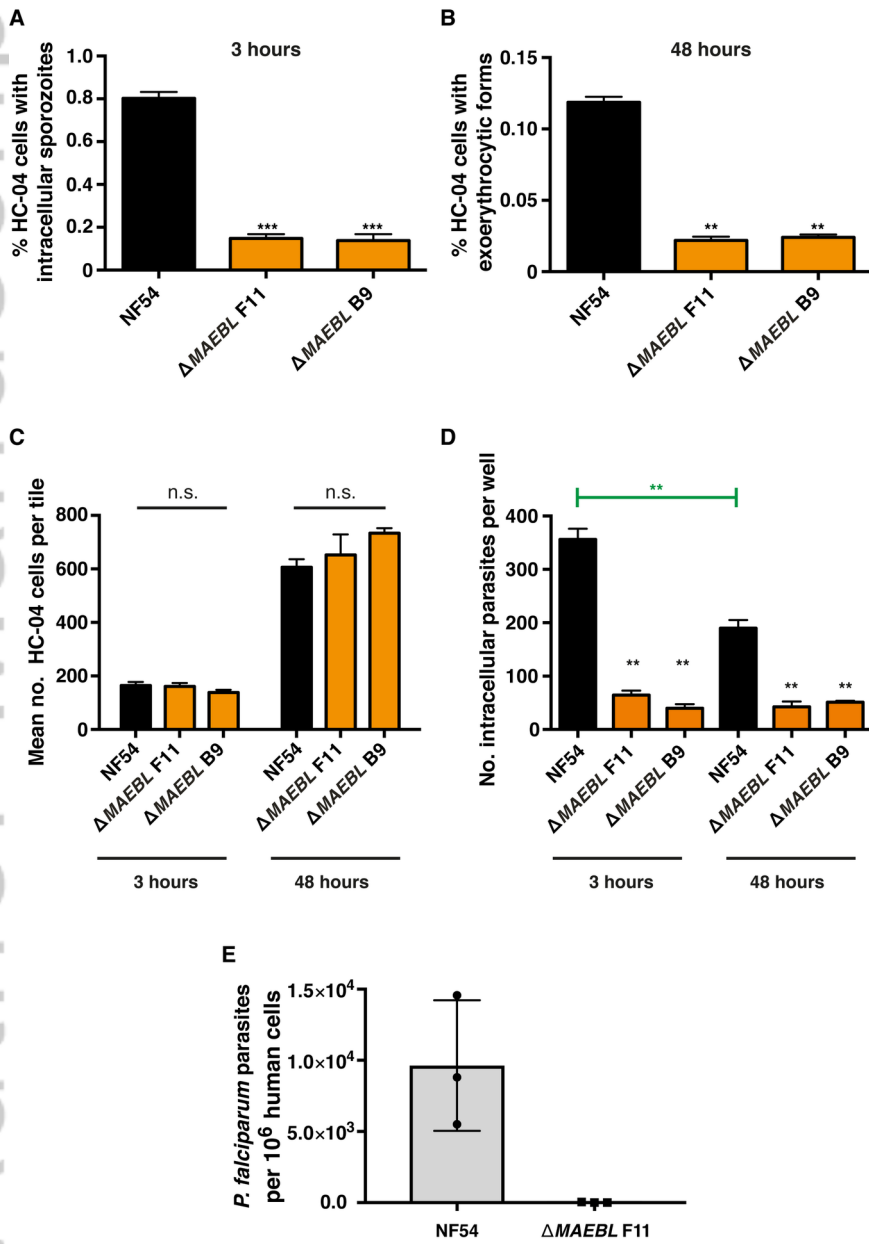
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Figure 4



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Figure 5



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