



## OPEN ACCESS

## EDITED BY

Abhisheka Bansal,  
Jawaharlal Nehru University, India

## REVIEWED BY

Joel Vega-Rodriguez,  
National Institute of Allergy and  
Infectious Diseases (NIH),  
United States  
Jun Miao,  
University of South Florida,  
United States  
Rapatbhorn Patrapuvich,  
Mahidol university, Thailand

## \*CORRESPONDENCE

Sudhir Kumar  
sudhir.kumar@seattlechildrens.org  
Stefan H. I. Kappe  
stefan.kappe@seattlechildrens.org

## SPECIALTY SECTION

This article was submitted to  
Parasite and Host,  
a section of the journal  
Frontiers in Cellular and  
Infection Microbiology

RECEIVED 26 July 2022

ACCEPTED 24 October 2022

PUBLISHED 25 November 2022

## CITATION

Kumar S and Kappe SHI (2022)  
PfHMGB2 has a role in malaria parasite  
mosquito infection.  
*Front. Cell. Infect. Microbiol.*  
12:1003214.  
doi: 10.3389/fcimb.2022.1003214

## COPYRIGHT

© 2022 Kumar and Kappe. This is an  
open-access article distributed under  
the terms of the [Creative Commons  
Attribution License \(CC BY\)](https://creativecommons.org/licenses/by/4.0/). The use,  
distribution or reproduction in other  
forums is permitted, provided the  
original author(s) and the copyright  
owner(s) are credited and that the  
original publication in this journal is  
cited, in accordance with accepted  
academic practice. No use,  
distribution or reproduction is  
permitted which does not comply with  
these terms.

# PfHMGB2 has a role in malaria parasite mosquito infection

Sudhir Kumar <sup>1\*</sup> and Stefan H. I. Kappe <sup>1,2,3\*</sup>

<sup>1</sup>Center for Global Infectious Disease Research, Seattle Children's Research Institute, Seattle, WA, United States, <sup>2</sup>Department of Pediatrics, University of Washington, Seattle, WA, United States, <sup>3</sup>Department of Global Health, University of Washington, Seattle, WA, United States

Differentiation of asexually replicating parasites into gametocytes is critical for successful completion of the sexual phase of the malaria parasite life cycle. Gametes generated from gametocytes fuse to form a zygote which differentiates into ookinetes and oocysts. The sporozoites are formed inside oocysts which migrate to the salivary glands for next cycle of human infection. These morphologically and functionally distinct stages require stage-specific gene expression *via* specific transcriptional regulators. The capacity of high mobility group box (HMGB) proteins to interact with DNA in a sequence independent manner enables them to regulate higher order chromosome organization and regulation of gene expression. *Plasmodium falciparum* HMGB2 (*PfHMGB2*) shows a typical L-shaped predicted structure which is similar to mammalian HMG box proteins and shows very high protein sequence similarity to *PyHMGB2* and *PbHMGB2*. Functional characterization of *PfHMGB2* by gene deletion (*Pfhmgb2*<sup>-</sup>) showed that knockout parasites develop normally as asexual stages and undergo gametocytogenesis. Transmission experiments revealed that *Pfhmgb2*<sup>-</sup> can infect mosquitoes and develop as oocyst stages. However, transmission was reduced compared to wild type (WT) parasites and as a consequence, the salivary gland sporozoites were reduced in number. In summary, we demonstrate that *PfHMGB2* has no role in asexual growth and a modest role in sexual phase development and parasite transmission to the mosquito.

## KEYWORDS

gametocyte, differentiation, transmission, mosquito, oocyst

## Introduction

*Plasmodium falciparum* (*Pf*) is a digenetic parasite which completes its life cycle in the human host and a female *Anopheles* mosquito. The development inside humans involves exoerythrocytic forms which transitions from asexual reproduction in hepatocytes to asexual schizogony in erythrocytes. During erythrocytic stage of infection, a small fraction of asexually replicating parasites commit to sexual development and differentiate into sexual stages called gametocytes. Inside the

mosquito midgut, the gametocytes taken up during an infectious blood meal get rapidly activated to form gametes, fuse to form short-lived zygotes which differentiate into motile ookinetes and penetrate the midgut epithelium wall to develop as oocysts. Oocyst form sporozoites which take residence in the salivary glands and are eventually injected into a new human host by bite.

The development and differentiation of *P. falciparum* sexual stages involves expression of specific transcripts (López-Barragán et al., 2011; van Biljon et al., 2019). The ApiAP2 family members have been studied for their role in transcriptional regulation (Kafsack et al., 2014; Shang et al., 2021; Singh et al., 2021). PfAP2-G is required for initial commitment of the asexual parasites into gametocytes and regulates additional transcriptional regulators (Llorà-Batlle et al., 2020). Sexual development also involves sex-specific transcript expression (Le Roch et al., 2004; Khan et al., 2005; Young et al., 2005) and translational repression of mRNAs via an RNA helicase DOZI (development of zygote inhibited) (Mair et al., 2006). Recent studies have also indicated the role of putative DNA and RNA binding proteins in regulating gamete fertility of *P. falciparum* and the rodent malaria *P. berghei* parasites (Russell et al., 2021; Kumar et al., 2022a; Kumar et al., 2022b).

Proteins belonging to the HMGB family (high mobility group box) (PFAM ID PF00505) are eukaryotic non-histone nuclear proteins which maintain the structure and function of chromosomes. HMGB proteins have multiple functions including DNA replication, transcription, and recombination (Travers, 2003). Mammalian HMGB1 is also secreted as a damage-associated molecular pattern (DAMP) molecule regulating inflammation and immune responses (Chen et al., 2022). Mammalian HMGB2 was initially identified as a male fertility regulator with high expression in lymphoid organs and testes (Ronfani et al., 2001). HMGB2 also plays a key role in spermatogenesis in turtles (Li et al., 2022). *P. falciparum* has four HMGB family proteins (PfHMGB1 - 4), which show transcriptional expression during the erythrocytic stages (López-Barragán et al., 2011). PfHMGB1 and PfHMGB2 proteins are expressed by both asexual and sexual stages, possess DNA binding affinities (Briquet et al., 2006), and are also secreted and are potent inducers of pro-inflammatory cytokines (Kumar et al., 2008). PfHMGB1 regulates gene expression via mediating the structural organization of the genome but is dispensable for asexual growth of parasites (Lu et al., 2021). Studies in rodent malaria parasite *P. yoelii* have demonstrated a role for HMGB2 in mosquito infection (Gissot et al., 2008) while in *P. berghei* it acts as an alarmin contributing to the cerebral malaria (Briquet et al., 2015) and confers long-lasting protection in a murine experimental cerebral malaria (Briquet et al., 2020).

PfHMGB2 shows higher expression in sexual stages (López-Barragán et al., 2011). It is reported to be refractory to gene

deletion (Lu et al., 2021), although a piggy bac mutagenesis study was able to report disruption and parasite survival with a compromised growth (Zhang et al., 2018). Since HMGB2 has a role in male fertility and spermatogenesis in other species (Ronfani et al., 2001; Li et al., 2022), we sought to determine its role in parasite sexual stage biology and transmission to the mosquito vector.

## Material and methods

### Reagents

Unless stated otherwise, the molecular biology reagents were purchased either from Millipore Sigma, USA or Thermo Scientific, USA. All oligonucleotides were purchased from Integrated DNA Technologies, USA.

### *P. falciparum* culture and transfection

*P. falciparum* NF54 and *Pfhmgb2*<sup>-</sup> parasites were cultured in accordance with standard procedures at 37°C and supplemented with “malaria” gas containing 5% O<sub>2</sub>/5% CO<sub>2</sub>/90% N<sub>2</sub>. Asexual cultures were set up at 5% hematocrit while gametocyte cultures were set up at 4% hematocrit using O<sup>+</sup> human red blood cells (Valley Biomedical, VA, US) and fresh medium was replenished daily. All cultures were maintained with complete RPMI media supplemented with either 0.5% AlbuMAX<sup>TM</sup> II (Thermo Scientific) medium as asexuals or 10% (v/v) type O<sup>+</sup> human serum (Valley Biomedical, VA, US or Interstate Blood Bank, TN, US) as gametocytes. Gametocyte cultures were set up at 1% ring stage parasitemia and were maintained in six well plates with a final volume of 5 mL using methods published elsewhere (Tripathi et al., 2020).

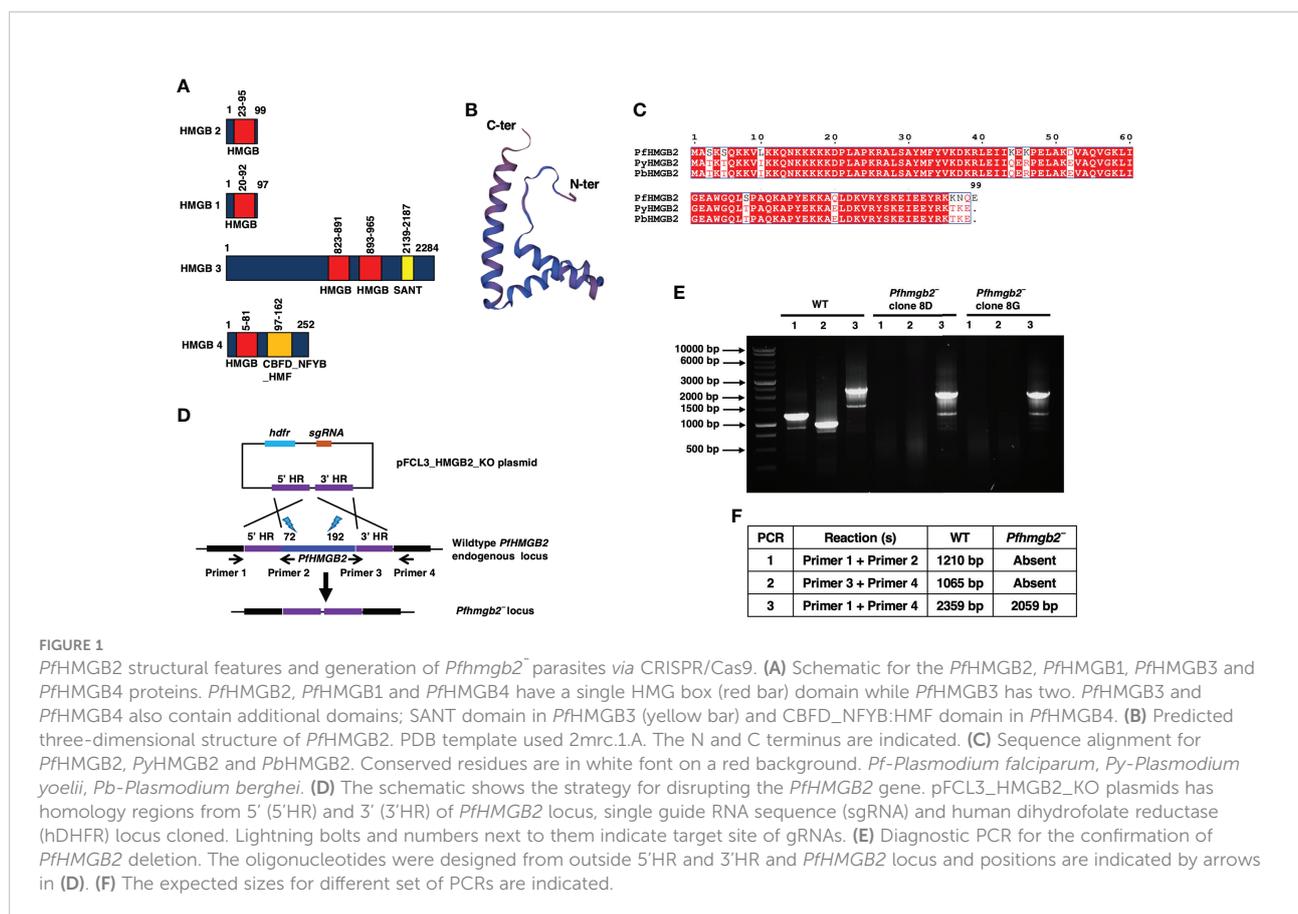
Oligonucleotides used in the generation and genotyping analysis of *P. falciparum* *Pfhmgb2*<sup>-</sup> parasites are mentioned in Table 1. Utilizing CRISPR/Cas9 strategy, the *PfHMGB2* locus (PlasmoDB gene identifier PF3D7\_0817900) was deleted by double crossover homologous recombination. The pFCL3\_HMGB2\_KO 1 plasmid was generated through the ligation of a 20-nucleotide guide RNA sequence along with the complementary regions of *PfHMGB2* flanking both ends of the open reading frame. Similarly, pFCL3\_HMGB2\_KO 2 was generated by cloning a different guide RNA sequence with same homology arms as pFCL3\_HMGB2\_KO 1. 100 µg each of these two plasmids were mixed and transfected into the NF54 ring stage parasites via electroporation at 310 V and 950 µF by using a Bio-Rad Gene Pulser II (Bio-Rad Laboratories, Hercules, CA). Transfected parasites were selected using 8 nM WR99210 (gifted by Jacobus Pharmaceuticals). *PfHMGB2* deletion on clones obtained using limiting dilution cloning was confirmed via genotyping PCR (Figures 1D–F). To further confirm the gene

TABLE 1 Oligonucleotides used in the study.

Oligonucleotides used for generation of *Pf*hmgb2<sup>-</sup> parasites

Oligo	Forward (5'-3')
<i>Pf</i> HMGB2 5'Homo For	TGCGGCCGCATATACATTTGTGTAGTTATATGTGTTACTATATATATGTTAAGTA
<i>Pf</i> HMGB2 5'Homo Rev	CCAACCGGGTATAGGCGCGCTGAACTGGTCATAATATTTCTGAAACAATGCATATA
<i>Pf</i> HMGB2 3'Homo For	AGGCGCGCCTATACCCGGGTTGGCATTCAACATATATATGAATAAATATATATGCGTG
<i>Pf</i> HMGB2 3'Homo Rev	TAAGTCGACCGATATAAECTTAATATTTATGTTTACACCTTAAATTATGTTTCA
<i>Pf</i> HMGB2 Guide 1 For	TATTGTAGGCAGACAAAGCTCTCTT
<i>Pf</i> HMGB2 Guide 1 Rev	AAACAAGAGAGCTTTGTCTGCCTAC
<i>Pf</i> HMGB2 Guide 2 For	TATTAATTGATAGGTGAAGCTTG
<i>Pf</i> HMGB2 Guide 2 Rev	AAACCAAGCTTACCTATCAATTT
<i>Pf</i> HMGB2 Geno5 For	TTAATGTTATAATTTTTTGTGTTTTCTTATTTATTTAAAAATTTAAACAATTTCTATAAAG
<i>Pf</i> HMGB2 Geno5 Rev	GCAACATCTTTTGTCTAATTTCTGGT
<i>Pf</i> HMGB2 Geno3 For	CAAAAGTACGATATTCAAAAGAAATAGAAGAATATAGAAA
<i>Pf</i> HMGB2 Geno3 Rev	CACATGCTATAAATATGTTACTATTTTTATATATCATAAAACATACCC
18s For	AACCTGGTTGATCCAGTAGTCATATG
18s Rev	CCAAAAATTGGCCTTGCAATTGTTAT
<i>Pf</i> HMGB2 ORF For	ATGGCTTCAAAATCTCAAAAGAAAGTATTAATAAAAAACAAAAAC
<i>Pf</i> HMGB2 ORF Rev	TTATTCTTGATTTTTCTTTCTATATTTCTTCTATTTCTTTT

Bold residues refer to additional nucleotides added to oligonucleotides for cloning and plasmid preparation.



deletion and absence of transcript, we prepared cDNA from WT and *Pfhmgb2*<sup>-</sup> parasites using QIAGEN kit following manufacturer's instruction and performed PCRs using *HMGB2* ORF oligonucleotides and 18s rRNA control oligonucleotides. Absence of a band for *HMGB2* in *Pfhmgb2*<sup>-</sup> confirmed gene deletion.

## Sequence analysis

*Plasmodium* DNA and protein sequences were retrieved from PlasmoDB (<http://plasmodb.org/plasmo/>). Domain analysis was done using SMART tool (<http://smart.embl-heidelberg.de/>).

## Measurement of asexual blood stage growth and gametocyte development

WT *PfNF54* and *Pfhmgb2*<sup>-</sup> parasites were synchronized at ring stages and were set up at 1% starting parasitemia and were maintained in 6-well plates as described above for comparative analysis of asexual blood stage development as well as gametocyte development. Asexual parasitemia was scored per 1000 erythrocytes after 48 and 96 hrs through Giemsa-stained thin blood smear microscopy. Gametocytemia per 1000 erythrocytes was scored on day 15 of *in vitro* culture, likewise through Giemsa-stained thin blood smear microscopy.

## Exflagellation, standard membrane feeding assay, oocyst and salivary gland sporozoite measurements

For analyzing exflagellation, equal volume of gametocytes from WT *PfNF54* and *Pfhmgb2*<sup>-</sup> were mixed separately with human type O<sup>+</sup> serum and O<sup>+</sup> RBCs (50:50) % (v/v) and incubated at room temperature for 15 min. Exflagellation was scored for WT *PfNF54* and *Pfhmgb2*<sup>-</sup> parasites *via* light microscopy by counting exflagellation centers in 10 optical fields of view at 40× magnification.

For SMFA, infectious blood meal was prepared by mixing stage V gametocytes for WT *PfNF54* or *Pfhmgb2*<sup>-</sup> with human serum and O<sup>+</sup> RBCs mixture (50:50) % (v/v) to achieve a final gametocytemia of 0.5%. Mosquitoes were fed as described in elsewhere (Tripathi et al., 2020). Following blood feeding, unfed mosquitoes were removed, and the rest were maintained for up to 20 days at 27°C, 75% humidity, and provided with 8% dextrose solution in 4-Aminobenzoic acid (PABA) water inside an incubator. *Anopheles stephensi* mosquitoes were dissected day 7 post blood meal for midguts and oocysts were enumerated under bright field microscope at 10× magnification. The mosquitoes were dissected day 14 post blood meal for

salivary glands and sporozoites were enumerated using Neubauer's chamber under bright field microscope at 40× magnification.

## Statistical analysis

Data collected was expressed as an average ± SD. Using unpaired two-tailed Student's t test or Nested one-way ANNOVA test with one-way ANOVA, the statistical differences in data were determined. GraphPad Prism 9 was used to calculate significances, with values of p < 0.05 being considered as statistically significant. Significance is represented in the figures as either ns- not significant, p > 0.05; \*p < 0.05; \*\*p < 0.01; or \*\*\*p < 0.001).

## Results

### Generation of *Pfhmgb2*<sup>-</sup> parasites

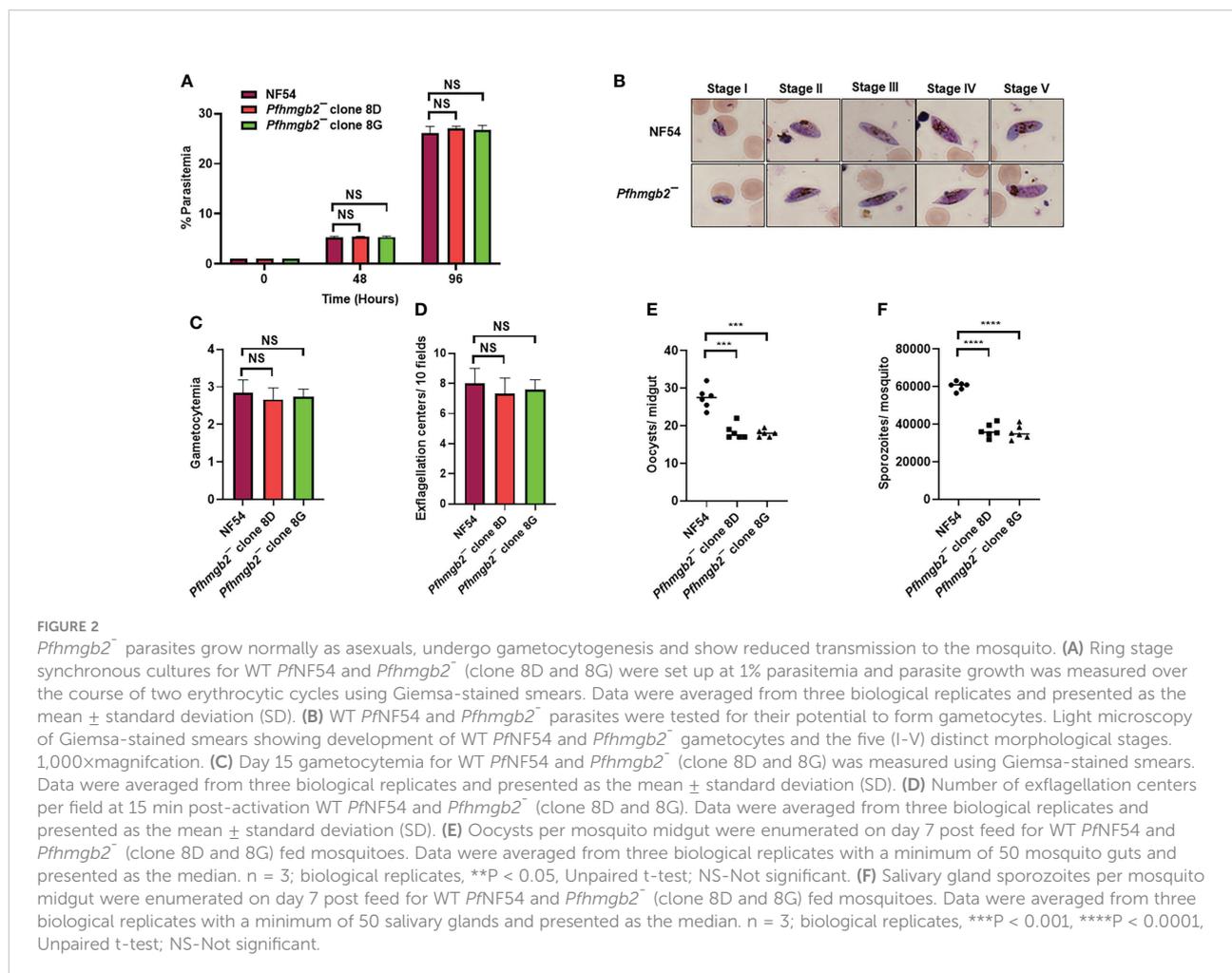
The *PfHMGB2* sequence was retrieved from PlasmoDB (<https://plasmodb.org/plasmo/app>) with gene identifier PF3D7\_0817900. HMG box proteins normally have two HMG boxes, but *PfHMGB2* is atypical 99 amino acid (aa) protein with a single HMG box (23-95 aa) (Figure 1A). A comparison of all four HMGB proteins from *P. falciparum* revealed the differences between their domains. Like *PfHMGB2*, *PfHMGB1* and *PfHMGB4* have a single HMG box domain while *PfHMGB3* contains two (Figure 1A). *PfHMGB3* and *PfHMGB4* also contain additional domains; SANT domain in *PfHMGB3* and Cbfd\_NFYB:HMF domain in *PfHMGB4* (Figure 1A). 3-dimensional structure prediction using SWISS-MODEL (<https://swissmodel.expasy.org/interactive/G2dB9b/models/>) showed that *PfHMGB2* has a conserved L-shaped structure like known eukaryotic HMG box proteins (Figure 1B). *PfHMGB2* shows very high similarity to rodent malaria parasite HMGB2 proteins (Figure 1C), indicating conservation of their function in the genus *Plasmodium*. Previous studies have indicated that *PfHMGB2* might be essential for asexual blood stages. This was assumed based on failure to obtain recombinant gene knockout parasites (Zhang et al., 2018; Lu et al., 2021). We however deleted the *PfHMGB2* gene using CRISPR/Cas9 mediated transgenesis (Figure 1D). Gene deletion parasites were confirmed by a set of diagnostic PCRs with oligonucleotide primers specific for the *PfHMGB2* locus and genomic regions 5' (upstream) and 3' (downstream) of the open reading frame (Figures 1D–F). Further PCRs were performed on cDNA prepared from WT *PfNF54* and *Pfhmgb2*<sup>-</sup> parasites for *HMGB2* ORF which confirmed deletion of the *PfHMGB2* locus (Supplementary Figure 1). To analyze the role of *PfHMGB2* in asexual blood stages, comparative growth assays were set up using two clones of *Pfhmgb2*<sup>-</sup> (clone 8D and 8G) alongside wildtype (WT) *PfNF54* parasites. The growth was monitored

over two replication cycles using Giemsa-stained thin smears prepared every 48-hr, which indicated that the growth rate of *Pfhmgb2*<sup>-</sup> was similar to WT NF54 (Figure 2A). These results demonstrate that HMGB2 is dispensable for asexual parasite replication.

## *Pfhmgb2*<sup>-</sup> parasites are transmissible to the mosquito vector and produce viable oocysts

We next analyzed the ability of *Pfhmgb2*<sup>-</sup> parasites to generate gametocytes. For this, WT and *Pfhmgb2*<sup>-</sup> (clone 8D and 8G) gametocytes were cultured *in vitro* as described elsewhere (Tripathi et al., 2020). Percent gametocytemia was scored for all the cultures on day 15 of *in vitro* culture using Giemsa-stained smears. This analysis revealed that *Pfhmgb2*<sup>-</sup> parasites were able to undergo gametocytogenesis (Figure 2B) and mature to stage V gametocytes and had similar gametocytemia as the WT NF54 parasites (Figure 2C). We

next analyzed the ability of *Pfhmgb2*<sup>-</sup> parasites to form motile male microgametes in exflagellation assays. For this, day 15 gametocyte cultures for WT and *Pfhmgb2*<sup>-</sup> parasites were activated by addition of O<sup>+</sup> human serum and a temperature drop from 37°C to room temperature (RT). After *in vitro* activation of the gametocytes, wet mounts were prepared and the number of exflagellation centers were evaluated using light microscopy at 40× magnification in ten random fields of view. We observed a similar number of exflagellation centers for *Pfhmgb2*<sup>-</sup> and WT *PfNF54* (Figure 2D), indicating that male gamete formation was normal in *Pfhmgb2*<sup>-</sup>. Previous studies on rodent malaria parasite *P. yoelii* have shown that *PyHMGB2* has a critical role in *Py* oocyst development in the mosquito vector (Gissot et al., 2008). Since *PfHMGB2* shows a very high degree of similarity and conservation with *PyHMGB2* (Figure 1B) and not with HMGB3 or HMGB4, we aimed at investigating a potential effect of *PfHMGB2* deletion on parasite transmission to *A. stephensi* mosquitoes. Infectious blood meals were prepared using standard methods for WT *PfNF54* and *Pfhmgb2*<sup>-</sup> stage V gametocytes and were fed to mosquitoes using standard



membrane feeders. Mosquitoes were dissected on Day 7 post blood meal for scoring midgut oocysts numbers. This revealed that *Pfhmgb2*<sup>-</sup> clones produced lesser average oocyst numbers in mosquitoes than WT *PfNF54* (Figure 2E). The median oocyst number in *A. stephensi* infected with the WT parasites was 28 while in *Pfhmgb2*<sup>-</sup> it was 18 (Figure 2E). Quantitative analysis of salivary gland sporozoites fourteen days post feed showed reduced number of sporozoites in mosquitoes fed with *Pfhmgb2*<sup>-</sup> (Figure 2F), likely caused by reduced numbers of oocysts.

Taken together, these results indicate that *PfHMGB2* is dispensable for gametocytogenesis and gametogenesis but has a modest role in parasite transmission to the mosquito vector or oocyst development.

## Discussion

In this study, we investigated the role of the *PfHMGB2* in asexual blood stage and sexual stage development. *Plasmodium* species harbor four HMGB proteins HMGB1-4 which are highly conserved among different parasite species (Briquet et al., 2006) and [PlasmoDB]. While all four proteins are expressed during asexual stages, *PfHMGB2* and *PfHMGB4* also show enhanced expression during gametocytogenesis (Lu et al., 2021), suggesting a role in sexual stage development.

HMGB proteins contain HMG box domain and are highly conserved throughout evolution. The HMG box domain is composed of ~80 aa folded in three  $\alpha$ -helices arranged in an L shape (Weir et al., 1993; Baxevanis and Landsman, 1995). In higher eukaryotes, many proteins contain HMG boxes, the majority of which are transcription factors and contain a single HMG-box (Thomas and Travers, 2001). Other proteins may contain up to 6 HMG-box domains such as for example Ubf1 (Russell and Zomerdijk, 2005). The HMGB proteins typically contain two boxes, A and B, along with basic N- and C-terminal extensions and a C-terminal acidic tail (Thomas and Travers, 2001). The HMG boxes A and B show differences in their sequences but have a well conserved L-shaped structure, and show differences in their DNA binding and bending abilities (Yoshioka et al., 1999). HMGB proteins may or may not act as transcription factors and do not have a DNA sequence preference. They typically assist other transcription factors by bending the cognate DNA sequence and altering the positioning of nucleosomes and thus controlling the level of transcription (Agresti and Bianchi, 2003; Längst and Becker, 2004). HMGB protein also function in chromosomal maintenance, possibly by telomere maintenance (Schumpfová et al., 2011; Polanská et al., 2012). The *HMGB1* gene deletion in mouse embryonic fibroblasts (MEFs) leads to chromosomal abnormalities, moderate shortening of telomere lengths, and lower telomerase activity compared to the WT MEFs, while *HMGB2* gene deletion (*hmg2*<sup>-</sup>) MEFs show elevated telomerase activity suggesting

their opposite effects on telomerase activity (Polanská et al., 2012). HMGB2 also has a major role in male fertility and spermatogenesis (Ronfani et al., 2001; Li et al., 2022). Recent reports suggest a role for *PfHMGB1* in the integrity of centromere/telomere-based chromosome organization and thus regulation of gene expression (Lu et al., 2021). Other *Pf* proteins (*PfHMGB2*-*HMGB4*) have not been functionally characterized so far.

Here we show that *PfHMGB2* shows a high degree of conservation with rodent malaria parasite orthologs. While *PfHMGB2* is expressed in asexual blood stages, its expression is elevated in stage V gametocytes (Briquet et al., 2006; Kumar et al., 2008). *PfHMGB2* contains an N-terminal extension followed by HMG box and a shorter c-terminal region and display a conserved L-shaped structure which is similar to HMG box proteins, suggesting its DNA binding properties. Previous studies have in fact demonstrated that both *PfHMGB1* and *PfHMGB2* possess DNA binding properties (Briquet et al., 2006). The differences in domain architecture between the four *P. falciparum* HMGB proteins suggest differences in their target sequences in the genome. *PfHMGB1* gene deletion parasites showed local chromatin alteration and dysregulated gene expression (Lu et al., 2021). For functional characterization of *PfHMGB2*, we created gene deletion parasites using CRISPR/Cas9 based gene editing. In contrast to a previous report (Lu et al., 2021), *PfHMGB2* could be deleted in our studies. Failure to obtain gene deletion parasites in previous study may be due to selection of the guide RNA which vary in their efficiency during CRISPR/Cas9 mediated transgenesis. This work revealed that, despite high expression of *PfHMGB2* in both asexual and sexual stages, it is not required for asexual blood stage replication and gametocyte development. We further demonstrated that *Pfhmgb2*<sup>-</sup> parasites undergo normal exflagellation, suggesting normal microgamete formation. Mosquito feed performed on WT *PfNF54* and *Pfhmgb2*<sup>-</sup> gametocytes revealed that *Pfhmgb2*<sup>-</sup> could transmit to mosquito vector, although the oocyst numbers and salivary gland sporozoite numbers were reduced in comparison to WT parasites. The *Pfhmgb2*<sup>-</sup> phenotype resembles the reduced transmissibility of *Pyhmgb2*<sup>-</sup> parasites (Gissot et al., 2008), although the defect was more severe in latter. This also suggests the similarity in HMGB2 function across different species in *Plasmodium*. The lack of a very strong observable phenotype in *Pfhmgb2*<sup>-</sup> parasites is surprising, given very high expression of *PfHMGB2* in mature gametocytes. We hypothesize that there could be redundancy in function of HMGB proteins in *Plasmodium*. There is a possibility that *PfHMGB1* can complement *PfHMGB2* function as both are expressed in asexual stages while *PfHMGB4*, which is highly expressed in sexual stages, can complement *PfHMGB2* during gametocyte stages and transmission to the mosquito. It is also possible that *PfHMGB2* along with *PfHMGB1* have role in immunomodulation which cannot be measured under

standard laboratory conditions for human infective parasites. This hypothesis is supported by the studies demonstrating that recombinant *Pf*HMGB1 and *Pf*HMGB2 are potent inducers of pro-inflammatory cytokines such as TNF $\alpha$  from mouse peritoneal macrophages (Kumar et al., 2008).

The sexual phase of *P. falciparum* life cycle represents a critical bottleneck and is required for transmission of the parasite from human host to mosquito. In this study, we show that *Pf*HMGB2 is dispensable for the asexual growth of parasite but has a role in parasite mosquito infection. Its conservation across various *Plasmodium* spp. strongly suggests it does have a significant role most likely in chromatin organization and gene expression. These hypotheses could be investigated in future studies.

## Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

## Author contributions

Conceptualization: SK. Methodology: SK. Investigation: SK. Visualization: SK, SHIK. Resources: SHIK. Supervision: SHIK. Writing-original draft: SK. Writing-review & editing: SK, SHIK. All authors contributed to the article and approved the submitted version.

## Funding

This work is supported by seed funds to S.H.I.K. by Seattle Children's via award number 24010119.

## References

- Agresti, A., and Bianchi, M. E. (2003). HMGB proteins and gene expression. *Curr. Opin. Genet. Dev.* 13, 170–178. doi: 10.1016/S0959-437X(03)00023-6
- Baxevasis, A. D., and Landsman, D. (1995). The HMG-1 box protein family: Classification and functional relationships. *Nucleic Acids Res.* 23, 1604–1613. doi: 10.1093/nar/23.9.1604
- Briquet, S., Boschet, C., Gissot, M., Tissandié, E., Sevilla, E., Franetich, J. F., et al. (2006). High-mobility-group box nuclear factors of plasmodium falciparum. *Eukaryot Cell* 5, 672–682. doi: 10.1128/EC.5.4.672-682.2006
- Briquet, S., Lawson-Hogban, N., Boisson, B., Soares, M. P., Péronet, R., Smith, L., et al. (2015). Disruption of parasite *hmg2* gene attenuates plasmodium berghei ANKA pathogenicity. *Infection Immun.* 83, 2771–2784. doi: 10.1128/IAI.03129-14
- Briquet, S., Lawson-Hogban, N., Peronet, R., Mécheri, S., and Vaquero, C. (2020). A genetically *hmg2* attenuated blood stage *p. berghei* induces crossed-long live protection. *PLoS One* 15, e0232183. doi: 10.1371/journal.pone.0232183
- Chen, R., Kang, R., and Tang, D. (2022). The mechanism of HMGB1 secretion and release. *Exp. Mol. Med.* 54, 91–102. doi: 10.1038/s12276-022-00736-w
- Gissot, M., Ting, L. M., Daly, T. M., Bergman, L. W., Sinnis, P., and Kim, K. (2008). High mobility group protein HMGB2 is a critical regulator of plasmodium oocyst development. *J. Biol. Chem.* 283, 17030–17038. doi: 10.1074/jbc.M801637200
- Kafsack, B. F., Rovira-Graells, N., Clark, T. G., Bancells, C., Crowley, V. M., Campino, S. G., et al. (2014). A transcriptional switch underlies commitment to sexual development in malaria parasites. *Nature* 507, 248–252. doi: 10.1038/nature12920
- Khan, S. M., Franke-Fayard, B., Mair, G. R., Lasonder, E., Janse, C. J., Mann, M., et al. (2005). Proteome analysis of separated male and female gametocytes reveals novel sex-specific plasmodium biology. *Cell* 121, 675–687. doi: 10.1016/j.cell.2005.03.027
- Kumar, S., Abatiyow, B. A., Haile, M. T., Oualim, K. M. Z., Leeb, A. S., Vaughan, A. M., et al. (2022a). A putative plasmodium RNA-binding protein plays a critical role in female gamete fertility and parasite transmission to the mosquito vector. *Front. Cell Dev. Biol.* 10, 825247. doi: 10.3389/fcell.2022.825247
- Kumar, S., Baranwal, V. K., Haile, M. T., Oualim, K. M. Z., Abatiyow, B. A., Kennedy, S. Y., et al. (2022b). *Falciparum* malaria parasite Male gametogenesis and

## Acknowledgments

The authors acknowledge continuous support and availability of the *Anopheles stephensi* mosquitoes provided by the Arthropod containment lab (ACL) I and II at CGIDR, Seattle Children's.

## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

## Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fcimb.2022.1003214/full#supplementary-material>

### SUPPLEMENTARY FIGURE 1

Confirmation of HMGB2 deletion. The cDNA was prepared from WT and *Pf**hmg2*<sup>-</sup> clone 8D parasites and PCRs were performed using the oligonucleotides designed from *HMGB2* open reading frame (ORF) and control 18s rRNA oligonucleotides. The band sizes for different set of PCRs are indicated.

female fertility and is critical for parasite transmission to the mosquito vector. *mBio* 13, e0057822. doi: 10.1128/mbio.00578-22

Kumar, K., Singal, A., Rizvi, M. M., and Chauhan, V. S. (2008). High mobility group box (HMGB) proteins of *Plasmodium falciparum*: DNA binding proteins with pro-inflammatory activity. *Parasitol. Int.* 57, 150–157. doi: 10.1016/j.parint.2007.11.005

Längst, G., and Becker, P. B. (2004). Nucleosome remodeling: one mechanism, many phenomena? *Biochim. Biophys. Acta* 1677, 58–63. doi: 10.1016/j.bbexp.2003.10.011

Le Roch, K. G., Johnson, J. R., Florens, L., Zhou, Y., Santrosyan, A., Grainger, M., et al. (2004). Global analysis of transcript and protein levels across the *Plasmodium falciparum* life cycle. *Genome Res.* 14, 2308–2318. doi: 10.1101/gr.2523904

Li, W., Zhu, J., Lei, L., Chen, C., Liu, X., Wang, Y., et al. (2022). The seasonal and stage-specific expression patterns of HMGB2 suggest its key role in spermatogenesis in the Chinese soft-shelled turtle (*Pelodiscus sinensis*). *Biochem. Genet.* 6, 2489–2502. doi: 10.1007/s10528-022-10229-0

Llorà-Batlle, O., Michel-Todó, L., Witmer, K., Toda, H., Fernández-Becerra, C., Baum, J., et al. (2020). Conditional expression of PfAP2-G for controlled massive sexual conversion in *Plasmodium falciparum*. *Sci. Adv.* 6, eaaz5057. doi: 10.1126/sciadv.aaz5057

López-Barragán, M. J., Lemieux, J., Quiñones, M., Williamson, K. C., Molina-Cruz, A., Cui, K., et al. (2011). Directional gene expression and antisense transcripts in sexual and asexual stages of *Plasmodium falciparum*. *BMC Genomics* 12, 587. doi: 10.1186/1471-2164-12-587

Lu, B., Liu, M., Gu, L., Li, Y., Shen, S., Guo, G., et al. (2021). The architectural factor HMGB1 is involved in genome organization in the human malaria parasite *Plasmodium falciparum*. *mBio* 12. doi: 10.1128/mBio.00148-21

Mair, G. R., Braks, J. A., Garver, L. S., Wiegant, J. C., Hall, N., Dirks, R. W., et al. (2006). Regulation of sexual development of *Plasmodium* by translational repression. *Sci. (New York N.Y.)* 313, 667–669. doi: 10.1126/science.1125129

Polanská, E., Dobšáková, Z., Dvořáčková, M., Fajkus, J., and Štros, M. (2012). HMGB1 gene knockout in mouse embryonic fibroblasts results in reduced telomerase activity and telomere dysfunction. *Chromosoma* 121, 419–431. doi: 10.1007/s00412-012-0373-x

Ronfani, L., Ferraguti, M., Croci, L., Ovitt, C. E., Schöler, H. R., Consalez, G. G., et al. (2001). Reduced fertility and spermatogenesis defects in mice lacking chromosomal protein Hmgb2. *Development* 128, 1265–1273. doi: 10.1242/dev.128.8.1265

Russell, A. J. C., Sanderson, T., Bushell, E., Talman, A. M., Anar, B., Girling, G., et al. (2021). Regulators of male and female sexual development critical for transmission of a malaria parasite. *bioRxiv*. doi: 10.1101/2021.08.04.455056

Russell, J., and Zomerdijk, J. C. (2005). RNA-polymerase-I-directed rDNA transcription, life and works. *Trends Biochem. Sci.* 30, 87–96. doi: 10.1016/j.tibs.2004.12.008

Schrumpfová, P. P., Fojtová, M., Mokroš, P., Grasser, K. D., and Fajkus, J. (2011). Role of HMGB proteins in chromatin dynamics and telomere maintenance in *Arabidopsis thaliana*. *Curr. Protein Pept. Sci.* 12, 105–111. doi: 10.2174/138920311795684922

Shang, X., Shen, S., Tang, J., He, X., Zhao, Y., Wang, C., et al. (2021). A cascade of transcriptional repression determines sexual commitment and development in *Plasmodium falciparum*. *Nucleic Acids Res.* 49, 9264–9279. doi: 10.1093/nar/gkab683

Singh, S., Santos, J. M., Orchard, L. M., Yamada, N., van Biljon, R., Painter, H. J., et al. (2021). The PfAP2-G2 transcription factor is a critical regulator of gametocyte maturation. *Mol. Microbiol.* 115, 1005–1024. doi: 10.1111/mmi.14676

Thomas, J. O., and Travers, A. A. (2001). HMGI and 2, and related 'architectural' DNA-binding proteins. *Trends Biochem. Sci.* 26, 167–174. doi: 10.1016/S0968-0004(01)01801-1

Travers, A. A. (2003). Priming the nucleosome: a role for HMGB proteins? *EMBO Rep.* 4, 131–136. doi: 10.1038/sj.embor.embor741

Tripathi, A. K., Mlambo, G., Kanatani, S., Sinnis, P., and Dimopoulos, G. (2020). *Plasmodium falciparum* gametocyte culture and mosquito infection through artificial membrane feeding. *J. Vis. Exp.* e41426. doi: 10.3791/61426

van Biljon, R., van Wyk, R., Painter, H. J., Orchard, L., Reader, J., Niemand, J., et al. (2019). Hierarchical transcriptional control regulates *Plasmodium falciparum* sexual differentiation. *BMC Genomics* 20, 920. doi: 10.1186/s12864-019-6322-9

Weir, H. M., Kraulis, P. J., Hill, C. S., Raine, A. R., Laue, E. D., and Thomas, J. O. (1993). Structure of the HMG box motif in the b-domain of HMGI. *EMBO J.* 12, 1311–1319. doi: 10.1002/j.1460-2075.1993.tb05776.x

Yoshioka, K., Saito, K., Tanabe, T., Yamamoto, A., Ando, Y., Nakamura, Y., et al. (1999). Differences in DNA recognition and conformational change activity between boxes a and b in HMGI protein. *Biochemistry* 38, 589–595. doi: 10.1021/bi981834l

Young, J. A., Fivelman, Q. L., Blair, P. L., de la Vega, P., Le Roch, K. G., Zhou, Y., et al. (2005). The *Plasmodium falciparum* sexual development transcriptome: a microarray analysis using ontology-based pattern identification. *Mol. Biochem. Parasitol.* 143, 67–79. doi: 10.1016/j.molbiopara.2005.05.007

Zhang, M., Wang, C., Otto, T. D., Oberstaller, J., Liao, X., Adapa, S. R., et al. (2018). Uncovering the essential genes of the human malaria parasite *Plasmodium falciparum* by saturation mutagenesis. *Sci. (New York N.Y.)* 360, eaap7847. doi: 10.1126/science.aap7847