Malaria parasites utilize two essential plasma membrane fusogens for gamete fertilization

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Received: 7 June 2022 / Revised: 28 September 2022 / Accepted: 1 October 2022 / Published online: 14 October 2022 © The Author(s), under exclusive licence to Springer Nature Switzerland AG 2022

Abstract
Cell fusion of female and male gametes is the climax of sexual reproduction. In many organisms, the Hapless 2 (HAP2) family of proteins play a critical role in gamete fusion. We find that Plasmodium falciparum, the causative agent of human malaria, expresses two HAP2 proteins: PfHAP2 and PfHAP2p. These proteins are present in stage V gametocytes and localize throughout the flagellum of male gametes. Gene deletion analysis and genetic crosses show that PfHAP2 and PfHAP2p individually are essential for male fertility and thereby, parasite transmission to the mosquito. Using a cell fusion assay, we demonstrate that PfHAP2 and PfHAP2p are both authentic plasma membrane fusogens. Our results establish nonredundant essential roles for PfHAP2 and PfHAP2p in mediating gamete fusion in Plasmodium and suggest avenues in the design of novel strategies to prevent malaria parasite transmission from humans to mosquitoes.

Keywords Plasmodium · Gametocyte · Cell fusion · Transmission · Microgamete · Macrogamete

Introduction
Malaria remains a major global health burden and a major cause of mortality and morbidity in developing countries worldwide. It is caused by Plasmodium parasites, with most deaths attributed to infection with Plasmodium falciparum. Malaria parasites are haploid Alveolates that reproduce asexually within two hosts: a vertebrate such as humans and a mosquito. Transmission from a human host to a mosquito vector requires sexual reproduction, which is essential for continuing the parasite life cycle. Sexual reproduction creates genetic diversity via recombination, allowing the parasite to rapidly adapt to its host and vector environment. Yet, sexual reproduction also constitutes a population bottleneck in the parasite life cycle that is vulnerable to disruption and the target of vaccine development efforts [1]. Plasmodium sexual stages form from a subset of asexually replicating parasites in the human blood stream and in P. falciparum, develop over a period of approximately 2 weeks into mature male and female gametocytes. They are then taken up by Anopheles mosquitoes during blood meal acquisition. Inside the mosquito midgut lumen, gametocytes are activated, rapidly differentiate into gametes, and egress from the infected red blood cells [2–4]. The male gametocyte undergoes three rapid rounds of DNA replication and forms eight flagellar (male) microgametes during its maturation [5]. The female gametocyte undergoes a marked reduction in cytoplasmic density and nuclear changes to form a single macrogamete [2, 3]. Male gametes exhibit motility through the blood meal and when encountering female gametes, attach and fuse, forming a short-lived diploid zygote [3, 5]. Zygotes differentiate into motile tetraploid ookinetes [6], penetrate the mosquito midgut epithelium and develop into oocysts. Within 2–3 weeks, oocysts differentiate into
haploid invasive sporozoites [7], which migrate to the salivary glands for transmission to a new host.

Gamete interactions that precede cell fusion involve four members of the Apicomplexan specific s48/45 domain 6-cysteine (6-cys) family of proteins—P48/45, P47, P230 and P230p [8–12]. Fertilization of the macrogamete by the microgamete then requires cell fusion. Yet, the molecular mechanisms underlying gamete fusion in Plasmodium and the involved fusogens are poorly understood. Eukaryotes utilize a wide array of proteins for different stages in fertilization, which can be distinct between vertebrates, invertebrates, plants, and protozoans [13–16]. One family of proteins involved in fertilization are the Hapless 2 (HAP2)/Generative Cell Specific 1 (GCS1) proteins which were identified in genetic screens [17, 18]. Moreover, HAP2(GCS1) from Arabidopsis was shown to be an authentic cell fusogen that can fuse heterologous cells [19–21]. HAP2 proteins show structural similarity to Class II viral fusion proteins and somatic animal fusogens [19–21]. These viral, somatic, and sexual fusogens are members of the fusexin superfamily and have a large extracellular region containing three conserved globular domains (domain I, domain II and domain III) [19]. As in viral fusogens, a conserved, ~46 amino acid residue region in domain II of HAP2 (the cd-loop) has been suggested to be involved in the fusion process by inserting itself into target membranes [19–21]. HAP2 gene knockouts in Chlamydomonas resulted in gametes of opposing mating types—mt(+) and mt(−)—to adhere to each other, but membrane fusion and thus fertilization was abrogated [22]. In the apicomplexan parasite Toxoplasma gondii, HAP2 also has a role in fertilization, and TgHAP2 knockout gametes show reduced fertilization [23]. Furthermore, two studies using the rodent malaria parasite Plasmodium berghei identified PbHAP2 and showed that it has a critical role in fertilization [22, 24]. However, the cell membrane fusogenic activity of PbHAP2 was not demonstrated.

In search for putative gamete fusogens of Plasmodium, we found that the human malaria parasite P. falciparum, as well as all other examined Plasmodium species, harbor two HAP2-like protein-encoding genes; the canonical PfHAP2 and a paralog, PfHAP2p. We show that in P. falciparum, HAP2 and PfHAP2p are expressed in gametocytes, activated male gametocytes, and microgametes. Interestingly, PfHAP2p was also expressed by females. In the absence of either protein, fertilization and transmission of the parasite to mosquitoes was completely blocked. Importantly, independent expression of PfHAP2 or PfHAP2p in mammalian cells mediated cell fusion, providing the first direct experimental evidence that these proteins serve as authentic cell membrane fusogens during Plasmodium sexual reproduction. This suggests PfHAP2 and PfHAP2p as attractive targets for developing transmission-blocking strategies against the most important human malaria parasites.

**Results**

**Two HAP2/GCS1-like proteins are encoded in Plasmodium genomes.**

To identify genes encoding HAP2/GCS1-like proteins in Plasmodium genomes, we searched for the HAP2 (PFAM10699) domain in the P. falciparum genome using PlasmoDB version 50 (https://plasmodb.org/plasmo/app). This revealed two distinct HAP2/GCS1-encoding genes in all Plasmodium species, PfHAP2 (PlasmoDB ID PF3D7_1014200) and PfHAP2p (paralog of HAP2, PlasmoDB ID PF3D7_0816300). In P. falciparum these are located on different chromosomes (chr.), namely PfHAP2 on chr. 10 and PfHAP2p on chr. 8. Domain analysis revealed a conserved domain architecture for both PfHAP2 and PfHAP2p (Fig. 1A, B), however, PfHAP2p lacked a predicted N-terminal signal peptide and possessed a longer cytoplasmic domain (Fig. 1A, B). HAP2 proteins showed structural similarity to eukaryotic/viral class II fusion proteins, particularly their domain II (DII) was predicted to contain a ~42-residue cd-fusion loop [19]. An amino acid sequence alignment of a part of DII regions from different HAP2 proteins: P. falciparum (PfHAP2 and PfHAP2p), Chlamydomonas reinhardtii (Cr), Leishmania major (Lm), Tribolium castaneum (Tc), Tetrahymena thermophile (Tt), Arabidopsis thaliana (At), Toxoplasma gondii (Tg), and Cryptosporidium muris (Cm), revealed conservation of several residues in the predicted cd-fusion loops in PfHAP2 and PfHAP2p which may be relevant to disulfide bridge formation, but multiple sequence differences were observed in different species (Fig. 1C). Sequence alignment of the HAP2 domain (PFAM10699) of PfHAP2 and PfHAP2p with HAP2 domains of Cr, Lm, Tc, Tt, At, Tg, and Cm was performed and also revealed sequence conservation (Fig. 1D). A further sequence analysis of the HAP2 domain (PFAM10699) of different Plasmodium species revealed high conservation (Figure S1A). 3D structural models for PfHAP2 (Fig. 1E) and PfHAP2p (Fig. 1F) were predicted and generated using a Phyre2 [25]. Several sections of the PfHAP2 and PfHAP2p models exhibited conserved structural folds similar to viral class II fusion proteins, AtHAP2 (PDB ID: 5OW3) (Fig. 1G) and CrHAP2 (PDB ID:5MF1) (Fig. 1H). Despite the similarities, however, sections of the PfHAP2 and PfHAP2p models were comprised of domains which are dissimilar to AtHAP2 and CrHAP2. Comparison of PfHAP2 and PfHAP2p predicted structures with structures from At and Cr, revealed key differences in the cd-loop region. While the cd-loop regions in At (Fig. 1G) and Cr...
Malaria parasites utilize two essential plasma membrane fusogens for gamete fertilization (Fig. 1H) were comprised of helical structures, the PfHAP2 cd-loop contained two antiparallel β strands (Figure S1B and Figure S1C) and the PfHAP2p cd-loop exhibited a flexible loop like structure (Figure S1D and Figure S1E). Key differences were also observed between the models of PfHAP2 and PfHAP2p (Fig. 1I) across all the domains, including domain I (Fig. 1J), domain II (Fig. 1K), cd-loop (Fig. 1L), and domain III (Fig. 1M). The domain II of PfHAP2p exhibited a 3-stranded antiparallel β sheet, which was completely absent in the HAP2 (Fig. 1K,L). Additionally, an α-helix of the Domain III from HAP2p was found to be replaced with a flexible loop like structure in HAP2 (Fig. 1M). While the C-terminal regions of HAP2 and HAP2p models also exhibited structural variations (Fig. 1N), an exact comparison was not made as most C-terminal residues of HAP2p could not be modelled.

PfHAP2 and PfHAP2p are expressed in gametocytes and gametes

To examine expression and localization of PfHAP2 and PfHAP2p in the sexual stages, two separate transgenic parasite lines were engineered to express GFP-tagged PfHAP2 (PfHAP2GFP) and mCherry-tagged PfHAP2p (PfHAP2pmCherry), using double-crossover homologous recombination (Figure S2). In addition, antibodies were generated against PfHAP2p using a KLH-conjugated peptide representing the cd-fusion loop (KLH-RQDDMFPLSFNYKKAKFKC).
Genotyping PCRs were performed to confirm the successful generation of transgenic \( P/HAP2^{GFP} \) parasites (Figure S2B and S2C) and transgenic \( P/HAP2^{p\,mCherry} \) parasites (Figure S2D and S2E). We then performed dual indirect immunofluorescence assays (IFAs) using anti-GFP antibody and anti-P230p antibody (an antibody specific to stage V male gametocytes), as well as anti-GFP antibody and anti-\( Pfg377 \) antibody (an antibody specific to female gametocytes). This revealed that \( P/HAP2 \) was expressed internally in male stage V gametocytes while in the microgametes, it was expressed throughout the flagellum (Fig. 2A). \( P/HAP2 \) expression was also detected in stage II, III and IV male gametocytes (Figure S3A). \( P/HAP2 \) expression was not detected in female stage V gametocytes (Fig. 2B). For further \( P/HAP2^{p}\) expression analysis, dual IFAs were performed using anti-mCherry and anti-tubulin antibody (an antibody specific to male stage V gametocytes and microgametes), as well as anti-mCherry antibody and anti-\( Pfg377 \) antibody. This revealed that \( P/HAP2^{p} \) was not expressed in stage II and III gametocytes (data not shown) but was expressed internally in male stage V gametocytes and throughout the flagellum (Fig. 2C). Unexpectedly, it was also expressed in female stage V gametocytes (Fig. 2D). \( P/HAP2^{p}\) expression was also detected in activated male gametocytes (Figure S3B) and activated female gametocytes (Figure S3C). \( P/HAP2^{GFP}\) parasites and \( P/HAP2^{p\,mCherry} \) parasites infected mosquitoes and formed oocysts similar to wildtype (WT) \( P/NF54 \) parasites, indicating that the addition of fluorescent tags to the C-termini of proteins did not interfere with their putative function (data not shown). These parasites were next used for creating a genetic cross (\( P/HAP2^{GFP} \times \) \( P/HAP2^{p\,mCherry} \)) to examine expression and localization of \( P/HAP2 \) and \( P/HAP2^{p} \) within the same parasite (Figure S4A). Genotyping PCRs were performed to confirm the successful generation of double transgenic \( P/HAP2^{GFP} \times \) \( P/HAP2^{p\,mCherry} \) parasites (Figure S4B, S4C and S4D). Dual IFAs using anti-GFP antibody and anti-mCherry antibody on \( P/HAP2^{GFP} \times \) \( P/HAP2^{p\,mCherry} \) gametocytes revealed that while \( P/HAP2 \) is expressed in stage II–stage V throughout the body of the gametocytes with maximum expression in stage V, \( HAP2^{p} \) is expressed in stage IV–stage V with maximum expression in stage V gametocytes (Fig. 2F). \( HAP2 \) and \( HAP2^{p} \) were both expressed in the same microgametes (Fig. 2F) and partially co-localized in the flagellum.

![Fig. 2](image-url)
**PfHAP2 and PfHAP2p are each critical for fertilization**

To determine the importance of *PfHAP2* and *PfHAP2p* in fertilization, we created gene deletion parasites using a CRISPR/Cas9 strategy (Figure S5). Two clonal lines each for *Pfhap2*<sup>−</sup> and *Pfhap2p*<sup>−</sup> individual gene knockouts were isolated by limiting dilution and successful gene deletions were confirmed by genotyping PCR (Figure S5). For phenotypic analysis of *Pfhap2*<sup>−</sup> and *Pfhap2p*<sup>−</sup> parasite clones, we performed asexual blood stage growth assays. Intraerythrocytic development was compared to WT *PfNF54* parasites over two replication cycles using Giemsa-stained culture smears. These assays showed that *Pfhap2*<sup>−</sup> and *Pfhap2p*<sup>−</sup> asexual stages did not exhibit any apparent growth defect (Fig. 3A). We next examined gametocyte formation and differentiation of *Pfhap2*<sup>−</sup> and *Pfhap2p*<sup>−</sup> parasites. No significant defect in gametocyte development and therefore, gametocytemia was observed on day 15 of gametocyte culture for *Pfhap2*<sup>−</sup> and *Pfhap2p*<sup>−</sup> when compared to gametocytemia for WT *PfNF54* control (Fig. 3B). To analyze microgamete formation, we assessed exflagellation, the rapid process by which microgametes form within activated male gametocytes and egress from the infected RBCs. *Pfhap2*<sup>−</sup> and *Pfhap2p*<sup>−</sup> stage V gametocytes were activated by adding human serum and a temperature shift to room temperature (RT). There were no significant differences observed in the number of exflagellation centers of *Pfhap2*<sup>−</sup> and *Pfhap2p*<sup>−</sup> and WT *PfNF54* control (Fig. 3C). Next, to ascertain the role of *PfHAP2* and *PfHAP2p* in fertilization, we analyzed zygote formation 15 hours post gamocyte activation. Anti-*Pfs25* antibody was used to stain WT *PfNF54*, *Pfhap2*<sup>−</sup> and *Pfhap2p*<sup>−</sup> activated cultures, which revealed no zygotes in *Pfhap2*<sup>−</sup> and *Pfhap2p*<sup>−</sup> parasite cultures. This stood in stark contrast to WT *PfNF54* control, which showed normal zygote formation (Fig. 3D). These results indicate that both *PfHAP2* and *PfHAP2p* are individually essential for zygote formation.

**Fig. 3** *PfHAP2* and *PfHAP2p* are each essential for fertilization. A Ring stage synchronous cultures for WT *PfNF54* and two different clones of *Pfhap2*<sup>−</sup> (clone E5 and F9) and *Pfhap2p*<sup>−</sup> (clone cl1 and cl3) were set up at 1% parasitemia and parasite growth was measured over the course of two erythrocytic infection cycles using Giemsa-stained culture smears. Data were averaged from three biological replicates and presented as the mean ± standard deviation (SD). B Number of exflagellation centers (emergence of microgametes) per field at 15 min post-activation. Data were averaged from three biological replicates and presented as the mean ± standard deviation (SD). C Zygotes were immunolabelled with anti-*Pfs25* antibody and counted in 50 optical fields in triplicate. Results are shown as mean ± SD. ND-Not detected.
** PfHAP2 and PfHAP2p are each essential for fertility of male gametes **

After ascertaining the critical roles of *PfHAP2* and *PfHAP2p* in fertilization, we examined the transmissibility of *Pfhap2<sup>−</sup>* and *Pfhap2p<sup>−</sup>* parasites to the mosquito vector. For this, stage V gametocytes of *Pfhap2<sup>−</sup>* and *Pfhap2p<sup>−</sup>* and WT *PfNF54* parasites were mixed with human serum and human red blood cells (RBCs) to prepare infectious blood meals for *Anopheles stephensi* mosquitoes. Analysis of mosquito midgut infection 7 days post blood meal showed no oocysts in the *Pfhap2<sup>−</sup>* and *Pfhap2p<sup>−</sup>* lines, demonstrating that they are each required for parasite transmission to mosquitoes (Fig. 4A). We next examined a sex-specific function for *PfHAP2* and *PfHAP2p* using genetic crosses with *Pfhap2<sup>−</sup>* and *Pfhap2p<sup>−</sup>* parasites in combination with transgenic parasite lines which formed either fertile female gametes only (*Pfcdpk4<sup>−</sup>*) [26] or fertile male gametes only (*Pfmacfet<sup>−</sup>*) [27]. WT *PfNF54*, *Pfhap2<sup>−</sup>*, *Pfhap2p<sup>−</sup>*, *Pfcdpk4<sup>−</sup>*, *Pfmacfet<sup>−</sup>* gametocytes were generated in vitro and cultures were first individually fed to *A. stephensi* mosquitoes on day 15 of in vitro culture. Oocyst numbers in the mosquito midgut were quantified on day 7 post blood meal and revealed that, as expected, only the WT *PfNF54* parasites infected mosquitoes and formed oocysts. For genetic crosses, gametocytes from the same cultures were mixed in pairs as follows: *Pfhap2<sup>−</sup>×Pfcdpk4<sup>−</sup>*, *Pfhap2p<sup>−</sup>×Pfcdpk4<sup>−</sup>*.

**Fig. 4** *PfHAP2* and *PfHAP2p* are critical for zygote formation and have male sex restricted function. A Mosquitoes were dissected on day 7 post feed and number of oocysts were measured per midgut. Data were averaged from three biological replicates with a minimum of 50 mosquito guts and presented as the mean ± standard deviation (SD). ND not detected. B Oocyst formation of WT *PfNF54*, *Pfhap2<sup>−</sup>*, *Pfhap2p<sup>−</sup>*, *Pfcdpk4<sup>−</sup>* and *Pfmacfet<sup>−</sup>* gametocytes. In vitro genetic crosses demonstrated that the *Pfhap2<sup>−</sup>* and *Pfhap2p<sup>−</sup>* showed productive cross-fertilization with the *Pfmacfet<sup>−</sup>* parasites (which produces functional males only), and not with *Pfcdpk4<sup>−</sup>* (which produces functional females only) (error bar indicates mean ± SD; n = 2)
individually have critical male-contributed roles in gamete fertility.

**PfHAP2 and PfHAP2p are functional cell fusogens**

*Arabidopsis thaliana* (At) HAP2/GCS1 was shown to be fusogenic in cell–cell and virus–cell fusion assays [21]. To determine whether *Pf*HAP2 and *Pf*HAP2p are authentic membrane fusogens, we tested whether they could mediate fusion of mammalian Baby Hamster Kidney (BHK) cells. We synthesized the complete codon-optimized coding regions of *Pf*HAP2 and *Pf*HAP2p and cloned them independently into pCI plasmids containing IRES controlled reporters that enable the quantification of cell content mixing (see Materials and Methods). We also appended the signal peptide (SP) of *Pf*HAP2 to the *Pf*HAP2p N-terminus because the endogenous protein lacks an apparent secretion signal. To determine whether mammalian cells were able to express *Pf*HAP2 and *Pf*HAP2p, we also included V5 tags in the C-termini of the predicted proteins and performed immunofluorescence microscopy, demonstrating that both proteins were independently expressed (Figure S5). Each plasmid contained a different reporter gene encoding a fluorescent protein with a distinct location. To measure *Pf*HAP2 cell fusion potential, we transfected BHK-cells either with pCI::*Pf*HAP2::GFPp (GFP in the cytoplasm) or pCI::*Pf*HAP2::H2B-RFP (RFP in the nucleus) and mixed the two populations. 42 h post-mixing, we quantified hybrid cells containing mixed green cytoplasm with red nuclei (Fig. 5). To measure *Pf*HAP2p cell fusion potential, we transfected BHK-cells with either pCI::*Pf*HAP2p::GFPp or with pCI::*Pf*HAP2p::H2B-RFP and mixed the two populations. Following 42 h post-mixing, we again quantified hybrid cells containing mixed green cytoplasm with red nuclei (Fig. 5). We found that both *Pf*HAP2 and *Pf*HAP2p induced cytoplasmic content mixing (i.e., cell fusion) to similar levels as the AtHAP2 (Fig. 5A–C). Thus, both *Pf*HAP2 and *Pf*HAP2p can independently mediate cell fusion. To observe the cell fusion events as they occur in the culture, we performed time-lapse imaging using myristoylated GFP as a membrane marker co-expressed with *Pf*HAP2 and imaged by spinning disk confocal microscopy. Figure 5D shows how two cells expressing *Pf*HAP22 recognized each other and fused to merge their cytoplasmic revealing cell–cell fusion (Movie S1).

**P. falciparum** HAP2 and HAP2p fuse BHK cells when homotypically expressed in both fusing cells

Cell fusion is a process that can be driven by a unidirectional mechanism in which only one of the fusing cells expresses the fusogenic protein, as is the case for known fusogens from enveloped viruses [28]. An alternative bidirectional mechanism requires the expression of the fusogens in both fusing membranes and this is characteristic of homotypic fusogens involved in fusion between somatic cells, for example, mediated by the EFF-1 or AFF-1 proteins from *C. elegans* [28–33] and Myomaker in vertebrate muscles [30]. In addition, the intracellular SNARE proteins are required to dock and fuse membranes and they must be present in both interacting membranes [36]. Our evidence from genetic crosses indicated that both *Pf*HAP2 and *Pf*HAP2p are male-contributed factors each critical for fertilization. To test whether *Pf*HAP2 and *Pf*HAP2p work unidirectionally or bidirectionally, we transfected BHK-cells with *Pf*HAP2 or *Pf*HAP2p together with a cytoplasmic GFP and mixed them with cells expressing only the vector encoding a nuclear H2B::RFP. Presence of multinucleated cells with only green cytoplasm would reveal fusion between cells expressing the fusogens; in contrast, the appearance of mixed cells with green cytoplasm and red nuclei would reveal unilateral fusion. As negative control we used BHK-GFPpnes cells mixed with BHK-H2B-RFP and we found mostly mononucleated cells and some binucleated dividing cells expressing only green cytoplasm (GFPpnes) or cells expressing only red nuclei (H2B-RFP). We found that both *Pf*HAP2 and *Pf*HAP2p expressing cells showed multinucleated cells expressing 2–8 nuclei with green cytoplasm (Fig. 6A, B). Similarly, AtHAP2 showed a similar result with 20–40% multinucleation (Fig. 6A, B). As a unilateral positive control, we used the Vesicular Stomatitis Virus-G glycoprotein and found an average of 80% multinucleation at neutral pH and 100% multinucleation at the optimal low pH. While the VSV-G expressing cells showed 50% multinucleated cells with mixed red nuclei and green cytoplasm at neutral pH and 100% after incubation at low pH, *Pf*HAP2, *Pf*HAP2p and AtHAP2 showed very few cells with mixed cytoplasmic levels similar to the negative control cells expressing only RFP or GFP (Fig. 6C). Moreover, expression of *Pf*HAP2 and *Pf*HAP2p in trans (different cells) or in cis (in the same cells) was unable to fuse cells and no mixing was observed (Fig. 6A, C). Thus, the *P. falciparum* HAP2 fusogens failed to fuse homologous mammalian BHK cells in a unidirectional (viral-like) fashion. The proteins must be present in both fusing cells.

**Discussion**

Fertilization is the central event during sexual reproduction when two gametes interact, and their plasma membranes fuse to form a zygote. This process generates an incipient genetically unique organism, creating genetic diversity in a population. The fusion of *Plasmodium* gametes during fertilization is an essential step in parasite transmission and occurs inside the mosquito vector. It creates novel parasite
**Fig. 5** *PfHAP2* and *PfHAP2p* mediate cell–cell fusion in mammalian cells. A–C BHK-21 cell–cell fusion was measured by content-mixing, indicated by the appearance of multinucleated cells containing blue nuclei (DAPI), magenta nuclei (H2B-RFP) and green cytoplasm (GFPnes). A Representative images of mixed cells. Scale bars 20 µm. B Cartoon of experimental design. C Quantification of content mixing. Cell–cell fusion was measured by content mixing, indicated by the presence of multinucleated cells containing magenta (H2B-RFP) or/and DAPI nuclei within cells with GFP cytoplasm (GFPnes). The mixing indices presented as means ± SEM of at least three independent trials. Comparisons by one way ANOVA followed by Bonferroni’s test. **p < 0.01; ***p < 0.0005; ****p < 0.0001. D Spinning disk confocal microscopy time-lapse images indicating the merging of two BHK cells expressing myristoylated-GFP (myrGFP) and *PfHAP2*. Time in hours:minutes. The green channel (GFP, white) and a merge of the DIC (grey) and Hoechst (magenta) are shown. White arrows and arrowheads indicate the fusing and fused cells, respectively. Scale bar 20 µm. (Refer to Supplementary Movie 1)
strains, some of them poised to have selective fitness advantages over their parents. However, the molecular mechanisms leading to fertilization are poorly understood in Plasmodium. Herein, we demonstrate that the human malaria parasite *P. falciparum* expresses two HAP2/GCS1 family members, *PfHAP2* and *PfHAP2p* and present genetic evidence that each is critical for male fertility only. We further demonstrate that *PfHAP2* and *PfHAP2p* can independently mediate cell membrane fusion and thus are authentic cell/gamete fusogens (Fig. 7).

HAP2/GCS1 family proteins are transmembrane proteins with large extracellular regions containing three globular domains [19–21]. HAP2 was initially identified in the flowering plant *A. thaliana* [17, 34] via a mutagenesis screen for male sterile lines, and in *Lilium longiflorum* pollen [18] as a sperm-specific protein involved in sperm-egg fusion. HAP2 proteins are the only gamete fusogens present in

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**Fig. 6** *PfHAP2* and *PfHAP2p* mediated cell–cell fusion is bidirectional in mammalian cells. Unidirectional BHK-21 cell–cell fusion was measured by content-mixing, indicated by the presence of multinucleated cells containing magenta nuclei (H2B-RFP) and green cytoplasm (GFPnes). **A** Images of mixed cells. Unilateral fusion was evaluated by mixing control cells expressing nuclear H2B-RFP (magenta) with cells expressing GFP with a nuclear export signal (GFPnes, green cytoplasm) only or together with *AtHAP2, PfHAP2, PfHAP2p, PfHAP2* and *PfHAP2p* (in cis) or VSV G incubated at neutral or low pH. Multinucleated cells labeled with DAPI only (arrowheads) or with both markers (arrows; green cytoplasm and magenta nuclei). Scale bars 20 µm. **B** Multinucleation index. **C** Quantification of content-mixing experiments. Bar chart showing means ± SEM of four independent experiments. Comparisons by one-way ANOVA followed by Dunnett’s test against the vector. ns non-significant, **p < 0.01, ****p < 0.0001. Source data are provided as a Source Data file.
three out of four eukaryotic kingdoms [19, 35–37]. They share similarity with class II viral fusion proteins [19–21]. One proposed model of action is that upon a trigger, a short conserved hydrophobic region (cd-loop) of HAP2 proteins becomes exposed on the male gamete plasma membrane and inserts into the female gamete plasma membrane while forming trimers, thereby overcoming the energetic barriers that are normally preventing membrane fusion [19, 20, 35]. This viral-like unilateral mechanism of action is different from the bilateral mechanism proposed for the related *C. elegans* EFF-1 and AFF-1. These somatic cell fusogens, structurally and functionally related to HAP2, act in the

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**Fig. 7** Model for *PfHAP2* and *PfHAP2p* function during *Plasmodium* gamete fusion. *PfHAP2* and *PfHAP2p* are expressed in male microgametes while *PfHAP2p* is also expressed in female macrogametes. The evidence from genetic crosses shows that both proteins are male-only contributed factors that are each essential for fertilization. This suggests that they are deployed by the microgamete to mediate membrane fusion with the macrogamete and that their mode of action is unidirectional (top panel). However, evidence from the cell fusion experiments shows that each *PfHAP2* and *PfHAP2p* must be present on both cells that engage in fusion (bottom panel) and that this bidirectional requirement is homotypic. *PfHAP2* and *PfHAP2p* might form dimeric or trimeric complexes during gamete fusion as has been shown for other membrane fusogens. This figure was created using Biorender.
respectively. Interestingly, the \textit{A.t}\textit{HAP2} also mediates bilateral cell fusion in BHK cells and in virus-cell fusion assays [21].

Previous studies identified a HAP2 ortholog in the rodent malaria parasite \textit{P. berghei} and showed that \textit{PbHAP2} functions in fertilization [22, 24], however, its precise molecular function remained undefined. We show here that \textit{Plasmodium} species possess two HAP2/GCS1 family proteins and demonstrate that they are authentic fusogens in a heterologous cell expression system and each is essential for fertilization in the human malaria parasite \textit{P. falciparum}. So far, only \textit{Dictyostelium discoideum} was shown to encode two paralogs of HAP2 related proteins, which are both critical for mating [38]. We found that \textit{PfHAP2} is expressed during male gametocytogenesis and in male microgametes, while \textit{PfHAP2p} was only detected in late-stage V gametocytes and microgametes but was also expressed in female gametocytes and macrogametes. Yet, in IFAs performed on live, non-permeabilized activated gametocytes/gametes, we did not observe staining with anti-\textit{PfHAP2p} antiserum. This suggests that \textit{PfHAP2} and \textit{PfHAP2p} are translocated to the plasma membrane and localize on the surface just-in-time before gamete fusion. Gene deletions for \textit{HAP2} and \textit{HAP2p} showed that neither of these proteins are essential for gametocyte formation and maturation. The formation and emergence (exflagellation) of microgametes are essential for gametocyte formation and maturation. The magnitude of \textit{PfHAP2} or \textit{PfHAP2p} cell fusion activity in this assay was comparable to the activity of \textit{A.t}\textit{HAP2}, indicating that the \textit{Plasmodium} proteins are potent membrane fusogens. Our genetic crosses clearly demonstrated a requirement for \textit{PfHAP2} and \textit{PfHAP2p} in the microgametes only. It is thus reasonable to assume that these fusogens are utilized unilaterally by the male to mediate fusion with the female (Fig. 7). In contrast however, the results in heterologous BHK cell fusion assays showed that in this in vitro system, the proteins cannot mediate unidirectional cell–cell fusion. Only the expression of either \textit{PfHAP2} or \textit{PfHAP2p} in both fusing cells mediated bidirectional cell–cell fusion (Fig. 7). It is of interest to point out that similar differences between genetic and cell fusion data with regard to directionality of the fusion event has also been observed for the \textit{A.t}\textit{HAP} [21]. These contradictory lines of evidence might be reconciled by the possibility that in the parasite, additional proteins of unknown identity on the female interact with HAP2 and HAP2p on the male, to enable unidirectional gamete fusion activity. The absence of such proteins in the BHK cells might, however, require HAP2 or HAP2p to be present on both fusing cells. Thus, the genetic and cell fusion data require conciliation with future experimentation. Because \textit{PfHAP2p} did not exhibit a predicted canonical SP that could mediate its entry into the parasite’s secretory pathway, it might require an escort partner protein. Alternatively, \textit{PfHAP2p} might contain a non-conventional SP that was not identifiable by bioinformatic prediction, or this protein might be trafficked to the parasite plasma membrane by an unconventional secretion mechanism described in \textit{Plasmodium} and other protozoans [39]. Once positioned on the microgamete plasma membrane, \textit{PfHAP2} and \textit{PfHAP2p} might form trimers (Fig. 7) as has been shown for HAP2 proteins of other organisms. To understand potential complex formation of \textit{PfHAP2} and \textit{PfHAP2p} will require direct future biochemical characterization in microgametes.

The domain organization of \textit{PfHAP2} and \textit{PfHA2p} is similar with an extracellular domain comprising domains I, II and III followed by a stem, membrane proximal region and transmembrane domain, which are typically present in HAP2/GCS1 family proteins. However, \textit{PfHAP2} and \textit{PfHAP2p} show predicted structural differences across domain I, domain II, and domain III. Intriguingly, different conformations were observed between the cd-loops of \textit{PfHAP2} and \textit{PfHAP2p}. HAP2p might have evolved multiple modes of membrane insertion to accommodate the diversity of membrane environments it has encountered during eukaryotic evolution [35]. Previously, cd-fusion loop differences of \textit{Arabidopsis}, \textit{Trypanosoma} and \textit{Chlamydomonas} HAP2 have been revealed by crystal structures [35]. It is also possible that different HAP2 cd-loops have differential lipid binding affinities towards different target membrane lipid compositions, and it will be very interesting to determine if \textit{PfHAP2} and \textit{PfHAP2p} have differential membrane lipid binding affinities in future studies. The recent discovery of HAP2-related Fusexin1 proteins in integrated mobile elements in archaea suggests novel mechanisms of horizontal gene transfer [40] that might help to explain the evolution...
of the fusexin family in eukaryotes, prokaryotes and the virome.

The transmission of sexual stages from the human host to the mosquito vector represents a major bottleneck in the malaria parasite life cycle [41]. This has been identified as a vulnerability in transmission and transmission blocking vaccines targeting surface molecules in gametocytes, gametes, zygotes and ookinetes have been proposed [42, 43]. Previous work has shown that immunizations with recombinant protein fragments encompassing amino acids 355–609 of PbHAP2 and 195–684 of PfHAP2 generated transmission blocking activity [44, 45]. Furthermore, antibodies targeting the cd-loop of PbHAP2 and PfHAP2 had shown transmission blocking activity with a 58.9% and 75.5% reduction in mosquito infection, respectively [46]. Very recently, antibodies targeting the domain III of PbHAP2 have also been shown to block fertilization in vitro and transmission to mosquitoes [47]. Thus, future studies could focus on identifying and targeting conserved antibody epitopes between PfHAP2 and PfHAP2p that could be simultaneously targeted to disrupt their respective fusogenic function.

In conclusion, we have shown herein that malaria parasites utilize two distinct HAP proteins, HAP2 and HAP2p, which can each independently mediate plasma membrane fusion. Each of these fusogens alone is critical for fertilization, and it remains to be determined whether they operate unidirectionally or bidirectionally in *Plasmodium* gamete fusion, together in a complex or serve individual and distinct functions. Furthermore, the signaling events that lead to the just-in-time surface-positioning and activation of PfHAP2 and PfHAP2p remain unknown and require future investigation.

**Methods**

**Reagents and primary antibodies**

All molecular biology reagents and oligonucleotides were purchased from Sigma–Aldrich. The following primary antibodies and antisera were used: mouse anti-GFP antibody (1:500, abcam, cat# ab1218 and cat# ab290); mouse anti-V5 antibody (1:500, Invitrogen, cat# R960-25); mouse anti-α-tubulin antibody (1:250, Sigma–Aldrich, cat# T9026); mouse anti-Pfβ (1:1, ATCC, cat# MRA-28), rat anti-mCherry clone 16D7 (1:100, Thermo Scientific cat# M11217); rabbit anti-Pfβ377 (1:500, kindly gifted by Professor Pietro Alano at Istituto Superiore di Sanità, Italy). The generation of polyclonal rabbit antisera against PfHAP2p (1:100) is described below.

**Sequence analysis and structure predictions**

*Plasmodium* DNA and protein sequences were retrieved from PlasmoDB (http://plasmodb.org/plasmo/) and aligned using Multalin (http://multalin.toulouse.inra.fr/multalin/) and Espript 3 (http://espiript.ibcp.fr/ESPr ipt/ESPr ipt/) programs. Signal peptide, transmembrane domains, and secondary structures were predicted using the SignalP-5.0 Server, then TMHMM Server v.2.0, and Phyre2 respectively. The models for *P. falciparum* HAP2 (all residues) and HAP2p (residues 1–515) were generated using the Phyre2 Server. The templates utilized by the Phyre2 intensive search algorithm were *A. thaliana* (PDB ID-5OW3), *C. reinhardtii* (PDB ID-5MF1) and *T. cruzi* (PDB ID-5OW4). For PfHAP2, 488 (55%) residues modelled at greater than 90% confidence, using *A. thaliana* (20% identity), *C. reinhardtii* (21% identity) and *T. cruzi* (25% identity) structures as the templates. For PfHAP2p, 409 (41%) residues modelled at greater than 90% confidence using re from *T. cruzi* (23% identity), *C. reinhardtii* (17% identity) and *A. thaliana* (19% identity) structures as the templates. The models generated were analyzed using ORION server to identify the fold and structurally similar molecules from the PDB. All structural comparisons and visualizations were performed using Pymol 2.0.

**Plasmodium falciparum strains and culturing**

*P. falciparum* NF54 was cultured as asexual blood stages according to standard procedures [48] and received medium changes every 24 h. Gametocytes were generated using O+ human RBCs (Valley Biomedical, VA, US) and O+ human serum. Cultures were kept at 37 °C and supplemented with complete RPMI medium supplemented with 0.05 g/L hypoxanthine, 0.3 mg/L L-glutamine and 10% (v/v) human serum. Cultures were kept at 37 °C and supplemented with gas containing 3% O2/5% CO2/92% N2.

**Generation of anti-PfHAP2p antibodies**

Peptide corresponding to amino acids 95–116 of PfHAP2p (QQDDMYFPFLSHFNYKKAFFKC), containing a fragment of the cd fusion loop, conjugated to carrier protein Keyhole limpet hemocyanin (KLH)—was synthesized by Biomatic Inc. (DE, US) and were used for immunization of animals. PfHAP2p antisera was generated by immunizing rabbits by Antagene Inc. (CA, US) and prescribes guidelines
for animal handling were followed. IgG purification and ELISA was performed by Antagene Inc.

**Generation of Pfhap2⁻, Pfhap2p⁻ and the creation of transgenic parasites expressing PfHAP2<sup>GFP</sup> and PfHAP2<sup>p<sub>mCherry</sub></sup>**

Oligonucleotide primers were used for the creation and analysis of *P. falciparum* hap2<sup>+</sup>, *P. falciparum* hap2p<sup>+</sup>, PfHAP2<sup>GFP</sup> and PfHAP2<sup>p<sub>mCherry</sub></sup> parasites are detailed in Table S1. Deletion of PfHAP2 (PlasmoDB identifier PF3D7_1014200) and PfHAP2p (PlasmoDB identifier PF3D7_0816300) was achieved based on the previously reported CRISPR/Cas9 strategy using pYC plasmid [51]. PfHAP2 and PfHAP2p were deleted using double crossover homologous recombination. Complementary regions of PfHAP2 or PfHAP2p upstream and downstream of the open reading frame were ligated into plasmid pFCL3 (generated by modification in pYC plasmid and described in [52]) as was the 20-nucleotide guide RNA sequence [51] resulting in the creation of a plasmid pFCL3_HAP2_KO and pFCL3_HAP2p_KO, respectively. Approximately 100 µg plasmid DNA was transfected into the *P. falciparum* NF54 strain using a previously published method [53] and selected using 8 nM WR99210.

Generation of double transgenic *PfHAP2<sup>GFP</sup> × PfHAP2<sup>p<sub>mCherry</sub></sup>* parasites

A genetic cross was generated between PfHAP2<sup>GFP</sup> and PfHAP2<sup>p<sub>mCherry</sub></sup> parasites using FRG NOD huHep mice [54] with human chimeric livers and *A. stephensi* mosquitoes as described elsewhere [55]. The gametocytes for *PfHAP2<sup>GFP</sup>* and *PfHAP2<sup>p<sub>mCherry</sub></sup>* were cultured in 6-well plates and were mixed in equal ratio and fed to *A. stephensi* mosquitoes where gametes for these two parasites would fuse and form hybrid progeny along with selfed parasites (Figure S4A). The salivary gland sporozoites from day 14 infected mosquitoes, which would contain double transgenic *PfHAP2<sup>GFP</sup> × PfHAP2<sup>p<sub>mCherry</sub></sup>* hybrid parasites, were injected intravenously (IV), and parasites were transitioned to in vitro culture day 7 post-injection. Parasites were cloned using limiting dilution and *PfHAP2<sup>GFP</sup> × PfHAP2<sup>p<sub>mCherry</sub></sup>* clonal parasites were confirmed by genotyping (Figure S4B, S4C and S4D). Clone C3 was used for performing IFAs for relative HAP2 and HAP2p expression analysis in the same parasite.

**Measurement of asexual blood stage growth and gametocyte development**

To compare asexual blood stage replication and growth between the WT *Pf*NF54, *Pfhap2⁻* and *Pfhap2p⁻* parasites, synchronized parasites were set up at an initial parasitemia of 1% at ring stages and cultured in 6-well plates as described above. Parasites were removed at 48 and 96 h. for preparation of Giemsa smears and parasitemia was scored per 1000 erythrocytes.

To compare gametocyte formation for WT *Pf*NF54, *Pfhap2⁻* and *Pfhap2p⁻* gametocytes were cultured as described above. Parasites were removed on day 15 of in vitro culture for preparation of Giemsa smears and gametocytemia was scored per 1000 erythrocytes.

**Measuring of exflagellation, standard membrane feeding assay (SMFA)**

Gametocytes of WT *Pf*NF54, *Pfhap2⁻* and *Pfhap2p⁻* were cultured as described above.Mosquitoes were reared and maintained on sugar water at 24 °C and 70% humidity. Gametocytes from all the cultures were analyzed for prevalence of stage V gametocytes using Giemsa smears. For assaying comparative exflagellation, equal volume of gametocytes from WT, *Pfhap2⁻* and *Pfhap2p⁻* were mixed with human serum and O⁺ RBCs (50:50) % (v/v) and incubated at room temperature for 10 min. Exflagellation was scored for WT *Pf*NF54, *Pfhap2⁻* and *Pfhap2p⁻* parasites via light microscopy by counting exflagellation centers in 10 random optical fields of view at 40× magnification in a bright field microscope.

For SMFA, stage V gametocytes were mixed with human serum and O⁺ RBCs mixture (50:50) % (v/v) to achieve a final gametocytemia of 0.5% and loaded on standard mosquito feeders. Mosquitoes were allowed to feed for approximately 25 min. Mosquitoes were dissected day 7 post-feeding and midguts were examined for oocysts by light microscopy.

**Comparative zygote formation assay**

For the analysis of zygote formation, mature day 15 WT, *Pfhap2⁻* and *Pfhap2p⁻* gametocytes were mixed with human serum and diluted to a final gametocytemia of 2%. Gametocyte cultures were activated in vitro by the addition of 100 µM xanthurenic acid followed by incubation for 30 min (macrogamete development) or 6 h. (zygote development) at room temperature (RT). An equal volume from each sample was loaded onto Teflon coated slides and
Immunofluorescence assay (described below) was performed with an anti-Pfs25 antibody. Macrogametes or zygotes for each sample were counted microscopically in a total number of 1000 of RBCs.

**Immunofluorescence assay (IFA) on Plasmodium falciparum parasites**

For IFAs of gametocytes and exflagellating gametes, cultures were fixed in 4% paraformaldehyde/0.0025% glutaraldehyde in PBS in a microcentrifuge tube by end-over-end rotation for 30 min. For each incubation step during IFA on gametocytes microcentrifuge tubes were kept on end-over-end rotation. For IFAs on zygotes, smears were prepared on Teflon coated slides and fixed with 4% paraformaldehyde/0.0025% glutaraldehyde solution for 30 min. Slides were kept in a humidity chamber for each step. Fixed parasites were washed twice with PBS and permeabilized using 0.1% Triton X-100/PBS solution for 10 min. Parasites were washed with PBS and blocked with 3%BSA/PBS for 45 min. Primary antiserum in 3%BSA/PBS was added to the parasites and slides or microcentrifuge tubes were incubated at 4 °C. Following three washes of 5 min each with PBS, appropriate secondary antibodies (Alex Fluor-488/594, Molecular Probes) were used at 1:500 dilution along with DAPI for 45 min at RT. Parasites were washed five times before mounting in ProLong Antifade (Thermo Fisher Scientific) mounting reagent. Images were obtained using a 100×1.4 NA objective 90 (Olympus) on a DeltaVision Elite High-Resolution Microscope (GE Healthcare Life Sciences).

**Mammalian cells culture**

BHK-21 cells (kindly obtained from Judith White, University of Virginia) were maintained in DMEM supplemented with 10% FBS (Biological Industries), 100 U/mL penicillin, 100 µg/mL streptomycin (Biological Industries), 2 mM l-glutamine (Biological Industries), 1 mM sodium pyruvate (Gibco), and 30 mM HEPES buffer, pH 7.3, at 37 °C with 5% CO₂. Transfections were performed using jetPRIME (Polyplus) according to the manufacturer’s instructions.

**PfHAP2 and PfHAP2p DNA constructs for expression in mammalian cells**

Full-length genes of PfHAP2 and PfHAP2p were synthesized after codon optimization for mammalian systems by IDT Inc. Since PfHAP2p does not have a predicted signal peptide, signal peptide of PfHAP2 was added at 5’ of codon optimized PfHAP2p sequence. These genes were then subcloned into corresponding backbones pCI::H2B-RFP and pCI::GFPpHAP2p which has a nuclear export signal (NES) at the C-terminus of GFP. The V5 tag was introduced at the C-terminus of PfHAP2 and PfHAP2p for immunofluorescence assays. See Supplementary Tables 1 and 2 for description of the plasmids and primers used.

**Plasmodium proteins expression in mammalian cells and immunofluorescence**

BHK-21 cells grown on 24-well tissue-culture plates with glass bottom or coverslips, were transfected when they reached 70% confluence using 1 µg pCI::PfHAP2::H2B-RFP or pCI::PfHAP2p::H2B-RFP. 18 h post-transfection, 20 µM 5-fluoro-2’-deoxyuridine (FdUrd) was added to the plates to block the cell cycle and 24 h later, the cells were fixed with 4% paraformaldehyde (PFA; EM grade, Bar Naor, Israel) in PBS, followed by incubation in 40 mM NH4Cl to block free aldehydes, permeabilized in 0.1% Triton X-100 and blocked in 1% Fetal Bovine Serum (FBS). We stained the cells with anti-V5 mAb (Invitrogen, 1:500), the secondary antibody was donkey anti–mouse coupled to Alexa Fluor 488 (Invitrogen, 1:500) and supplemented with 1 µg/mL DAPI [21]. Micrographs were obtained using wide-field illumination using an ELYRA system S.1 microscope and a Plan-Apochromat 63X NA 1.4 objective (Zeiss); images were recorded with an iXon + EMCCD camera (Andor).

**Cell fusion assay in mammalian cells**

BHK-21 cells at 70% confluence were transfected with 1 µg pCI::ArHAP2::GFPpHAP2, pCI::ArHAP2::H2B-RFP, pCI::PfHAP2::GFPpHAP2, pCI::PfHAP2::H2B-RFP, pCI::PfHAP2p::GFPpHAP2 and pCI::PfHAP2p::H2B-RFP respectively. Control cells were transfected with pCI::GFPpHAP2 or pCI::H2B-RFP (vectors). Four hours after transfection, the cells were washed 4 times with DMEM with 10% serum (Invitrogen), 4 times with PBS, and detached using Trypsin (Biological Industries). The transfected cells were collected in Eppendorf tubes, resuspended in DMEM 10% FBS, and counted. Equal amounts of H2B-RFP and GFPpHAP2 cells were mixed and seeded in glass-bottom plates (12-well black, glass-bottom #1.5H; CELLvis) and incubated at 37 °C in 5% CO₂. 18 h after mixing 20 µM FdUrd was added to the plates and 24 h later, the cells were fixed with 4% PFA in PBS. Nuclei were stained with 1 µg/mL DAPI. Micrographs were obtained using wide-field illumination using an ELYRA system S.1 microscope and a Plan-Apochromat 20X NA 0.8 lens (Zeiss); images were recorded with an iXon + EMCCD camera (Andor). The GFP + RFP mixing index was calculated as the number of nuclei in mixed cells, GFPpHAP2p cytoplasm with red (H2B-RFP) and blue (DAPI) nuclei out of the total number of nuclei of fluorescent cells in contact and expressed as percentage.
Live imaging of BHK cells expressing PfHAP2

BHK-21 cells were plated on 15 mm glass bottom plates (Wuxi NEST Biotechnology Co., Ltd.) and transfected with 1 µg pCI:: PfHAP2::H2B-RFP together with 0.5 µg myristoylated-GFP (myr-GFP) [56]. 18 h after transfection, the nuclei were stained with 2 µg/mL Hoechst dye for 10 min at 37 °C and the cells washed once with fresh medium. Time-lapse microscopy was performed using a spinning disc confocal microscope (CSU-X; Yokogawa Electric Corporation) with an Eclipse Ti and a Plan-Apochromat 20X (NA, 0.75; Nikon) objective. Images in differential interference contrast, DAPI and green channels were recorded every 4 min in different positions of the plate using high gain and minimum laser exposure. Time lapse images were captured with an iXon 3 EMCCD camera (Andor Technology). Image analyses were performed in MetaMorph (v7.8) (Molecular Devices) and ImageJ (v1.53c) (National Institutes of Health).

Cytoplasmic content mixing to determine unidirectional versus bidirectional mechanism

BHK-21 cells were transfected (as explained above) with 1 µg of each plasmid: pCI::GFPnes; pCI::ArHAP2- V5::GFPnes; pCI PfHAP2-V5::GFPnes; pCI::P/HAP2p::GFPnes; pCI::VSV-G::GFPnes; in 35 mm plates. Four hours after transfection, the cells were washed, detached, counted, and mixed with an equal number of pCI::H2B-RFP (empty vector) expressing cells, and mixed cells were incubated as previously described. In all cases, 18 h after mixing, 20 µM FdUrd was added to the plates, and 24 h later, the cells were fixed with 4% paraformaldehyde diluted in PBS (without calcium and magnesium). Nuclei were stained with 1 µg/mL DAPI. Images were obtained using wide-field illumination with an ELYRA system S.1 microscope as described above. The GFP + RFP mixing index was calculated as the number of nuclei in mixed cells, green cytoplasm (GFPnes) with red (H2B-RFP) and blue (DAPI) nuclei out of the total number of nuclei in fluorescent cells in contact. The multinucleation indexes were defined as the ratio between the number of nuclei in multinucleated cells (N_m) and the total number of nuclei in mononucleated cells and expressing cells that were in contact (N_c) but did not fuse, using the following equation: % multinucleation = N_m/(N_c + N_m) × 100. For pCI:: VSV-G::GFPnes mixed with pCI::RFP at low pH, 2 h before fixing DMEM medium was changed by HMS buffer pH5.5, incubated for 5 min, and changed again by DMEM and incubated for additional 2 h.

Cell fusion assay by content mixing to determine unidirectional versus bidirectional mechanism when both proteins are expressed in the same cell

BHK-21 cells were co-transfected with 1 µg pCI PfHAP2-V5::GFPnes and 1 µg pCI::PfHAP2::GFPnes in 35 mm plates. Four hours after transfection, the cells were washed, detached, counted, and mixed with an equal number of pCI::H2B-RFP (empty vector) expressing cells; a mixing experiment was performed as described before.

Cell fusion assay by content mixing for interaction in trans

BHK-21 cells were transfected (as explained above) with 1 µg pCI::PfHAP2::GFPnes or pCI::PfHAP2::RFP in 35 mm plates. Four hours after transfection, the cells were washed, detached, counted, and mixed, and the GFP + RFP mixing index was calculated as described before.

Statistical analysis

All data are expressed as mean ± SD. Statistical differences were determined using one-way ANOVA with post hoc Bonferroni multiple comparison test or unpaired two-tailed Student’s t test, as indicated in the figure legends. Values of p < 0.05 were considered statistically significant. Significances were calculated using GraphPad Prism 8 and are represented in the Figures as follows: ns, not significant; *p < 0.05; **p < 0.01; ***p < 0.001.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1007/s00018-022-04583-w.

Author contributions

SK, SHIK and BP planned and analyzed experiments and wrote the manuscript. SK, CV, MTH, XL, KF, NGB, SYK; BAA, ASL, and NMC performed experiments. AMV, SHIK and BP contributed to preparation of figures. The final manuscript was edited and approved by all authors.

Funding

This work was funded by the NIH P01 AI127338 to A.M.V. and S.H.I.K., Seattle Children’s internal seed funding to S.H.I.K and the Israeli Science Foundation F.I.R.S.T No. 2327/19 to B.P. Funders had no role in study design, data collection, and interpretation or the decision to submit the work for publication.

Data availability

All data are presented in the manuscript. Derived data supporting the findings of this study are available from the corresponding author on request.

Declarations

Conflict of interest

The authors declare no competing financial and non-financial interests.
Ethical statement The animal experiments, which involved antisera generation in rabbits, were performed at Antagene Inc. (CA, US) and prescribed guidelines were followed.

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