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The role of MICOS in organizing mitochondrial cristae in malaria parasites

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eLife Assessment

This study provides **valuable** insights into mitochondrial cristae organization in Plasmodium falciparum, particularly in the context of its divergent MICOS composition. The authors present **convincing** evidence, supported by phenotypic and morphological analyses, that cristae junction maintenance can be uncoupled from de novo cristae formation, reinforcing an emerging model of mitochondrial inner membrane organization. Notably, the absence of Mic10 alongside an enlarged and divergent MICOS complex highlights an intriguing evolutionary adaptation, although further characterization of the complex would strengthen the study's overall significance.

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Abstract

The malaria parasite mitochondria represent the only tractable, naturally occurring system in which cristae, the characteristic invaginations of the inner mitochondrial membrane, are known to be formed *de novo*. In traditional model organisms, the central complex involved in cristae organization is the mitochondrial contact site and cristae organizing system (MICOS). However, whether MICOS has an active role in the initial formation of cristae remains unclear. We identified two putative *Plasmodium falciparum* MICOS components, *Pf*MIC19 and *Pf*MIC60 and show that both genes are dispensable in asexual blood stages, gametocytes, and mosquito stages, albeit with a mild reduction in oocyst numbers. Immunofluorescence microscopy and electron tomography of gametocytes lacking either or both MICOS orthologues showed aberrant mitochondrial morphology and abnormal cristae, with a marked reduction of crista junctions. Thus, by utilizing the unique properties of *P. falciparum*, we confirmed the involvement of *Pf*MIC19 and *Pf*MIC60 in the organization of crista junctions, while providing evidence that MICOS is not required for the initial formation of cristae.

Introduction

The mitochondrion is a double membrane-bound organelle found in most eukaryotes. Its primary function is to generate ATP through oxidative phosphorylation. The mitochondrion bears an outer mitochondrial membrane (OMM), which is a selectively permeable barrier to the cytoplasm, and an inner mitochondrial membrane (IMM), which separates the mitochondrial matrix from the intermembrane space. The IMM is divided into two distinct domains: the cristae membrane and the inner boundary membrane. The cristae membrane is highly convoluted and rich in ATP synthase and the electron transport chain complexes, thus forming a specialized area responsible for oxidative phosphorylation. The inner boundary membrane hosts proteins and protein complexes responsible for exchange of metabolites, protein translocation, and mitochondrial

fusion (1-3). Both membrane regions are connected at the crista junctions. Crista junctions have been mainly described as tubular channels that connect the cristae membrane to the inner boundary membrane (4-6). Though, in organisms with lamellar cristae, such as yeast, crista junctions have also been identified as more elongated structures, also called slit-shaped crista junctions (7).

Crista junctions are known to be organized by a multi-subunit protein complex, the mitochondrial contact site and cristae organizing system (MICOS) (3, 8-11). Together with proteins of the OMM, MICOS is also part of the mitochondrial intermembrane space bridging complex that mediates contacts between the IMM and OMM (12-14). In humans, MICOS is organized into two protein sub-complexes integrated in the cristae membrane, the MIC10 and MIC60 sub-complexes, consisting of four and three different components, respectively (14-16). Disruption of either of the two MICOS sub-complexes has been shown to lead to loss of crista junctions and detachment of crista membranes. This generates aberrant cristae, followed by an atypical mitochondrial morphology (10, 15, 17). MIC60 is one of the key components of MICOS implicated in generating the negative curvature at the base of the cristae membrane (18, 19). MIC60 consists of an N-terminal transmembrane domain, responsible for anchoring it to the IMM, a coiled-coil domain and a mitofilin domain at the C-terminal end of the protein. These two C-terminal domains are essential to form and stabilize crista junctions through the tetramerization of MIC60 (19-21).

Strikingly, the mitochondrion of *Plasmodium falciparum*, the most virulent causing agent of human malaria, departs from the yeast or human model of mitochondria (22). Asexual blood-stage parasites (ABS) have a single acristate mitochondrion that harbours low levels of respiratory chain complex components and ATP synthase assemblies (23, 24). During development to the sexual, mosquito transmissible stages, also called gametocytes, the single mitochondrion separates into a set of 4-8 mitochondria (25) in which canonical mitochondrial processes, such as tricarboxylic acid cycle and oxidative phosphorylation gain relevance (23, 26-28). This is accompanied by a large increase in expression of ATP synthase and all relevant electron transport chain complexes but also by the *de novo* emergence of bulbous cristae (23, 25).

Even though mutation-induced aberrations of mitochondrial cristae morphology in *P. falciparum* have been sporadically reported (29), details on the molecular underpinnings of the bulbous shape and *de novo* biogenesis of cristae in *P. falciparum* gametocytes remain largely unknown. While a recent study in a related apicomplexan parasite, *Toxoplasma gondii*, has shown that the bulbous morphology is associated with highly divergent and large ATP synthase assemblies (5), the mechanisms involved in forming and maintaining stable crista junctions have yet to be investigated. Very little is known about any MICOS-like complex components or their potential roles in governing membrane architecture at the crista junction (29). Prior research has suggested PF3D7_1007800 as a putative orthologue of *MIC60* in *P. falciparum* (*PfMIC60*) based on sequence homology to the characteristic mitofilin domain, but until now, no other subunits of MICOS have been identified in *P. falciparum* (14). It is noteworthy that even though sequence homology and composition of MICOS diverges between distantly related eukaryotic clades, overall architecture is comparable to the opisthokont models (22).

Although MICOS has been described as an organizer of crista junctions, its role during the initial formation of nascent cristae has not been investigated. In this study, we used a combination of experimental genetics, high-resolution fluorescence microscopy, electron microscopy and electron tomography to elucidate the involvement of the parasite's MICOS in cristae organization, with clear distinction from cristae formation.

Results

Identification of putative *Pf*MICOS components

To further investigate previously reported sequence homology of the MIC60 mitofilin domain to the *Pf*MIC60 candidate PF3D7_1007800 (14), we compared publicly available AlphaFold predictions of the protein sequence of the canonical MIC60 and our candidate sequence (Figure 1A, C and Figure S1A, C). In addition to the putative mitofilin domain in *Pf*MIC60, we identified a putative

lipid binding domain and a coiled coil domain of similar length as described for opisthokonts MIC60. Interestingly, the annotated open reading frame of *Pf*MIC60 (UniProt Q8IJW5) is much larger than the *Saccharomyces cerevisiae* MICOS counterpart (UniProt A6ZZY0). The AlphaFold prediction of the full annotated open reading frame of PfMIC60 and ScMIC60 suggests the conserved domains (mitofilin, coiled coil and lipid binding domain) to make up the C-terminal half of PfMIC60. Even though confidence scores within the N-terminal half of the predicted molecule are low, Topology-based Evolutionary Domains (TED) identified a domain consisting of a stack of two parallel β-sheets in PfMIC60. Because this domain is predicted solely from computational analysis, both its actual existence in the native protein and its biological function remain unknown.

Since the *P. falciparum* genome lacks obvious orthologues of additional MICOS components, we re-examined our publicly available gametocyte-derived complexome profiling datasets (23). *Pf*MIC60 did not enter standard mass range gels but in samples separated on specialized, high molecular mass gels with lower acrylamide percentage, we found *PfMIC60* consistently comigrates with PF3D7_1108800 at an apparent mass of 4-5 MDa (Figure 1E, Supplementary Information 1) (23). PF3D7_1108800 is conserved across the *Plasmodium* genus (OG7_0323199) (30) and, while it lacks clear sequence homology outside of that, the AlphaFold prediction features a conserved CC-helix-CC-helix (CHCH) domain also found in MIC19, a known interactor of MIC60 in opisthokonts (Figure 1B, D and Figure S1B, C). Each of the two α-helices of the CHCH domain contains the conserved Cx9C motif that is characteristic for MIC19 CHCH domains in opisthokonts (Figure 1F). Based on these similarities and the comigration pattern, we propose to provisionally designate PF3D7_1108800 as *Pf*MIC19.

*Pf*MIC60 and *Pf*MIC19 are expressed during gametocyte stages

To study the function of *Pf*MIC19 and *Pf*MIC60 as possible MICOS components, we analysed their expression profile and localization in ABS and gametocytes. Therefore, we generated two transgenic parasite lines, *mic19-HA* and *mic60-HA* in which we introduced a *3xHA-GlmS* tag at the 3' end of the respective genes. The transgenic lines also contain a mitochondrially targeted mScarlet for visualization of the organelle and protein co-localization (Figure S2A). Correct integration and absence of wild-type (WT) parasites was confirmed through diagnostic PCR (Figure S2B). Western blot analysis of mixed ABS and mature gametocyte samples revealed that *Pf*MIC19 (19.7 kDa) and *Pf*MIC60 (120 kDa) are undetectable in acristate ABS but expressed in the cristate sexual blood stages (Figure 1G). As a positive control for mitochondrial localization, we used the MitoRed line (31), which expresses the mitochondrially targeted mScarlet from a silent intergenic locus. Unfortunately, attempts to localise *Pf*MIC19-HA and *Pf*MIC60-HA using conventional immunofluorescence microscopy and ultra-expansion microscopy were unsuccessful, likely due to their naturally low abundance (Figure S2C-D) highlighted by our western blot analysis (Figure 1G).

*Pf*MIC19 and *Pf*MIC60 are not essential for *P. falciparum in vitro* life-cycle progression

To investigate the essentiality of *Pf*MIC19 and *Pf*MIC60 during the *P. falciparum* life cycle, we generated the knockout parasite lines *mic19*$^{-}$ and *mic60*$^{-}$ in which the respective genes were replaced by a cytoplasmic green fluorescent protein (GFP) (Figure S2A). In addition, we generated the *mic19/60*$^{-}$ double-knockout line in which *PfMIC60* was replaced by a cytoplasmic red fluorescent protein (mCherry) in the *mic19*$^{-}$ background (Figure S3A). Diagnostic PCR confirmed proper integration and absence of WT parasites in all the generated lines (Figure S2B and Figure S3B). To assess parasite viability, we performed a standard growth assay on ABS. Parasite replication was followed for two replication cycles, revealing that ABS of *mic19*$^{-}$, *mic60*$^{-}$, and *mic19/60*$^{-}$ grow at a comparable rate to parental parasites (NF54) (Figure 2A). Interestingly, *mic19*$^{-}$, *mic60*$^{-}$, and *mic19/60*$^{-}$ also produced mature gametocytes with no apparent delay. To verify their viability, we performed exflagellation assays that revealed no difference in maturation compared to the NF54 parental control (Figure 2B). In addition, gametocytes were fed to

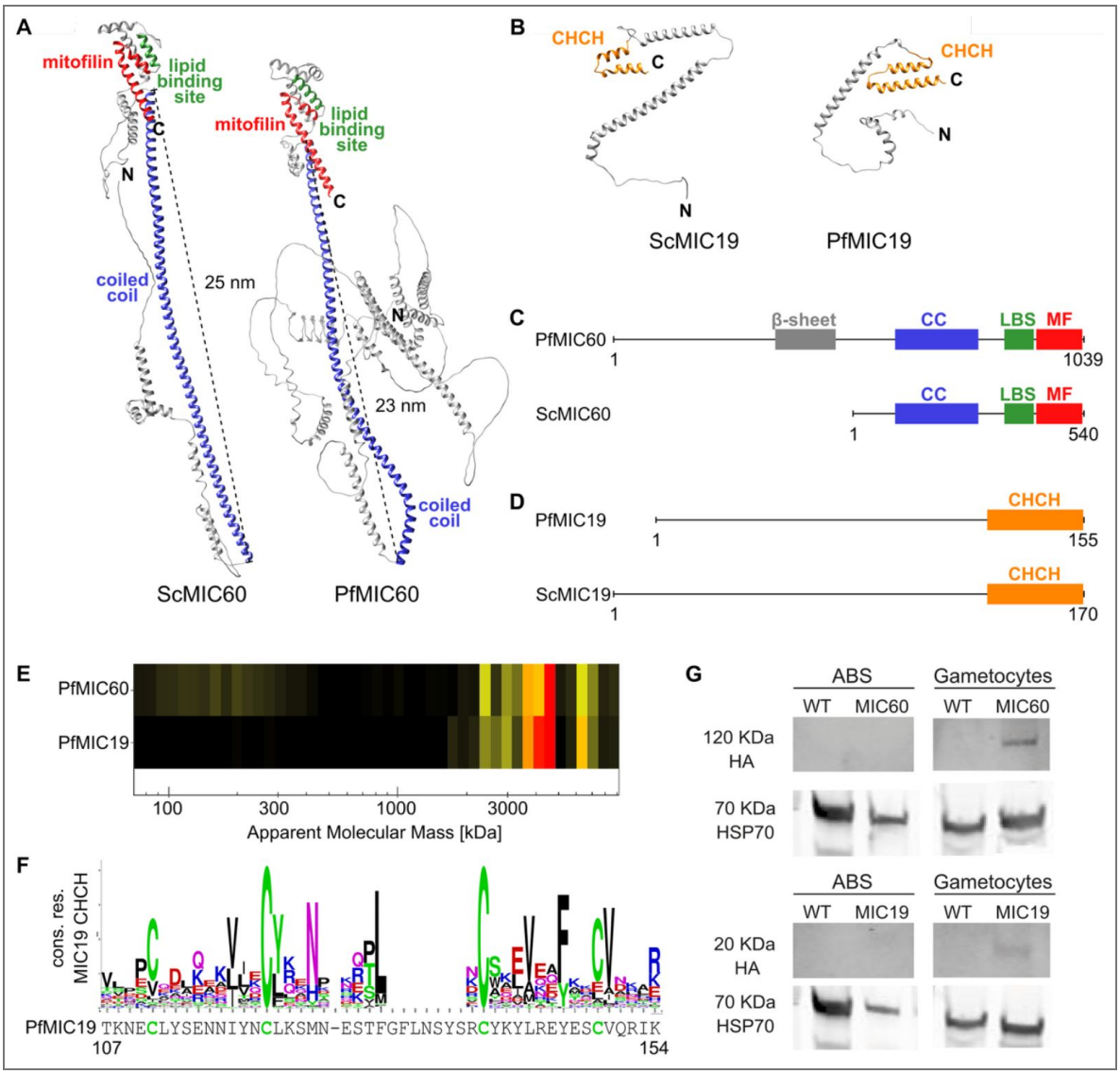

Figure 1. Identification of PfMICOS candidates based on predictive structural similarities, complexome profiling and the characteristic expression patterns expected for cristae modulators.

A) AlphaFold predictions of ScMIC60 (AFDB ID: AF-A6ZZY0-F1-v4) and PfMIC60 (AFDB ID: AF-Q8IJW5-F1-v4)(32, 33). TED consensus domain predicted mitofilin domain (red), lipid binding site (green) and coiled coil domain (blue, predicted length annotated) are highlighted (34). N- and C-terminus are annotated. (local pLDDT confidence information in Figure S1). B) AlphaFold predictions of ScMIC19 (AFDB ID: AF-P43594-F1-v4) and PfMIC19 (AFDB ID: AF-Q8IIR5-F1-v4)(32, 33). TED predicted CC-helix-CC-helix (CHCH) domain is highlighted (34). N- and C-terminus are annotated. (local pLDDT confidence information in Figure S1). C) Schematic overview of the TED predicted domain architecture of ScMIC60 and PfMIC60. The additional domain annotated for PfMIC60 consists of ten antiparallel β-sheets of unknown function. D) Schematic overview of the TED predicted domain architecture of ScMIC19 and PfMIC19. E) Migration of PfMIC19/PfMIC60 across fractions of a 3-16% BN-PAGE gel. Corresponding apparent molecular mass of gel fractions is indicated on the x-axis. Relative abundance across fractions is indicated through a heatmap. Co-occurrence in gel fractions is indicative of a shared protein complex. F) Sequence logo derived form a MAFFT alignment of non-redundant protein sequences from MIC19 annotated entries listed on NCBI (MMseqs2 filtered at threshold 0.9, aligned sequences listed in Supplementary Information 2). Letter height indicates information content. Amino acid frequencies are shown by relative letter size. The characteristic set of two CX9C motifs found in MIC19 is conserved in PfMIC19 (sequence shown below). G) Western blot analysis of ABS and mature gametocytes of MIC19-HA and MIC60-HA. Tagged proteins were detected with rat anti-HA (PfMIC19 and PfMIC60, top) and rabbit anti-HSP70 was used as a loading control (bottom).

mosquitoes through standard membrane feeding assays. All lines infected mosquitoes and we observed a reduction in oocyst numbers in *mic19*⁻, *mic60*⁻, and *mic19/60*⁻ compared to NF54 (Figure 2C and Figure 2D). The high variability encountered in the standard membrane feeding assays, though, partially obstructs a clear conclusion on the biological relevance of the observed reduction in oocyst numbers. Nevertheless, sporozoite production was confirmed for all generated knockout lines, indicating that *Pf*MIC19 and *Pf*MIC60 are not essential for the parasites to complete mosquito-stage development (Figure S5). In summary, under *in vitro* culture conditions, *Pf*MIC19 and *Pf*MIC60 are dispensable for survival and development of ABS and gametocytes. Absence of these MICOS components is also compatible with progression through mosquito-stage development.

Knockout of *PfMIC60* and *PfMIC19* impairs mitochondrial morphology in gametocytes

Next, we investigated the impact of *Pf*MIC19 and *Pf*MIC60 absence on mitochondrial morphology. Due to the lack of detectable protein expression in ABS, we focused on mature gametocytes, where cristae are abundant and protein expression was detected in western blot. We stained mitochondria of parental NF54 line, single and double knockout line mature gametocytes with MitoTracker and used alpha tubulin II as a known and previously confirmed male marker (35). To analyse the variability of normal mitochondrial morphology in the NF54 background, we quantified the imaging data using the Mitochondria-Analyzer plug-in for Fiji (36, 37). MitoTracker signals were classified into three categories: (1) tubular, when only one/two elongated and highly branched mitochondrial signals were identified per cell body; (2) intermediate, when multiple partially branched mitochondrial signals were observed; and (3) rounded-up, when mitochondrial signals appeared smaller, more spherical, and dispersed (Figure 3A). Note that light microscopy does not resolve individual mitochondria if they are in close contact with each other (25). Nevertheless, the analysis revealed that mitochondrial morphology of both male and female gametocytes was partially altered in all knockout lines, characterized by a predominance of rounded-up and intermediate mitochondria, and a marked reduction of tubular networks. (Figure 3B, Figure S6A). In addition to the rounded-up appearance of mitochondria, we also observed a dispersion of the mitochondrial network into spatially separated clusters. Wild-type cells contain a single compact cluster of closely apposed tubular mitochondria, whereas knockout cells display multiple smaller, dispersed mitochondrial clusters. Based on the number of individually identifiable MitoTracker signals within a cell, we determined the relative dispersion of the mitochondrial network. Even though not significant, the data indicates a trend towards increased network dispersion in all knockout lines (24%, 21%, and 23% in *mic19*⁻, *mic60*⁻, and *mic19/60*⁻) (Figure S6B). The single identified mitochondrial signals were ~30% smaller (Figure S6C), more spherical (36%, 24%, and 20%) (Figure 3C), and less branched (50%, 55%, and 40%) (Figure S6D) compared to the parental NF54 line. In addition, total mitochondrial volume per cell was reduced in all knockout lines with 15%, 17%, and 13%, respectively (Figure 3C). Interestingly, deletion of both genes did not exacerbate the mitochondrial phenotype compared to the single knockouts. Finally, no difference was detected in MitoTracker signal intensity, possibly indicating that the membrane potential is not affected in the single nor double knockouts (Figure S6E). In conclusion, while NF54 cells contain mainly tubular mitochondria, *mic19*⁻, *mic60*⁻, and *mic19/60*⁻ gametocytes have more intermediate and rounded-up mitochondria.

*Pf*MIC60 and *Pf*MIC19 are involved in shaping mitochondrial cristae

To assess the role of *Pf*MIC19 and *Pf*MIC60 in cristae morphology, we performed transmission electron microscopy (TEM) on mature gametocyte of *mic19*⁻, *mic60*⁻, *mic19/60*⁻, and the parental NF54. Two-dimensional analysis of thin sections revealed cristae deformations in all knockout lines (Figure 4A). We did not observe differences in cristae morphology between male and female parasites (Figure 4A and Supplementary Information 2). Length, diameter, and area occupied by individual cristae from over 30 mitochondrial cross-sections per sample were measured (Figure 4B-D). Note that such measurements do not indicate the true total length or

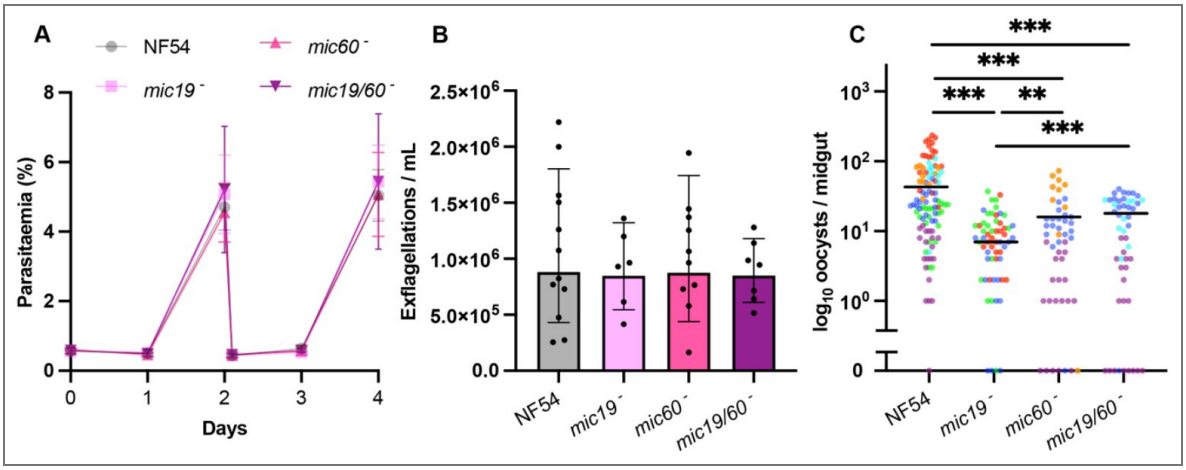

Figure 2. Consequences of deletion of PfMIC60 and PfMIC19 on differentiation and survival of P. falciparum.

A) Growth curve of NF54, mic19^{-}, mic60^{-}, and mic19/60^{-} ABS over time (in days). (n = 3 for NF54, mic19^{-}, mic60^{-} and n = 2 for mic19/60^{-}). B) Exflagellation assay of NF54, mic19^{-}, mic60^{-}, and mic19/60^{-}. (n >= 6). Statistical significance calculated with one-way ANOVA with Sidàk correction for full comparison between all cell lines. C) Infection of A. stephensi mosquitoes with NF54, mic19^{-}, mic60^{-}, and mic19/60^{-} in 6 independent experiments indicated in different colours. Means (black line): NF54 = 43, mic19^{-} = 7.0, mic60^{-} = 15.0 and mic19/60^{-} = 17.1. Statistical significance was assessed with Wald statistics and indicated as follows: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. Oocyst count of 0 was plotted at the x-axis for visualisation purposes.

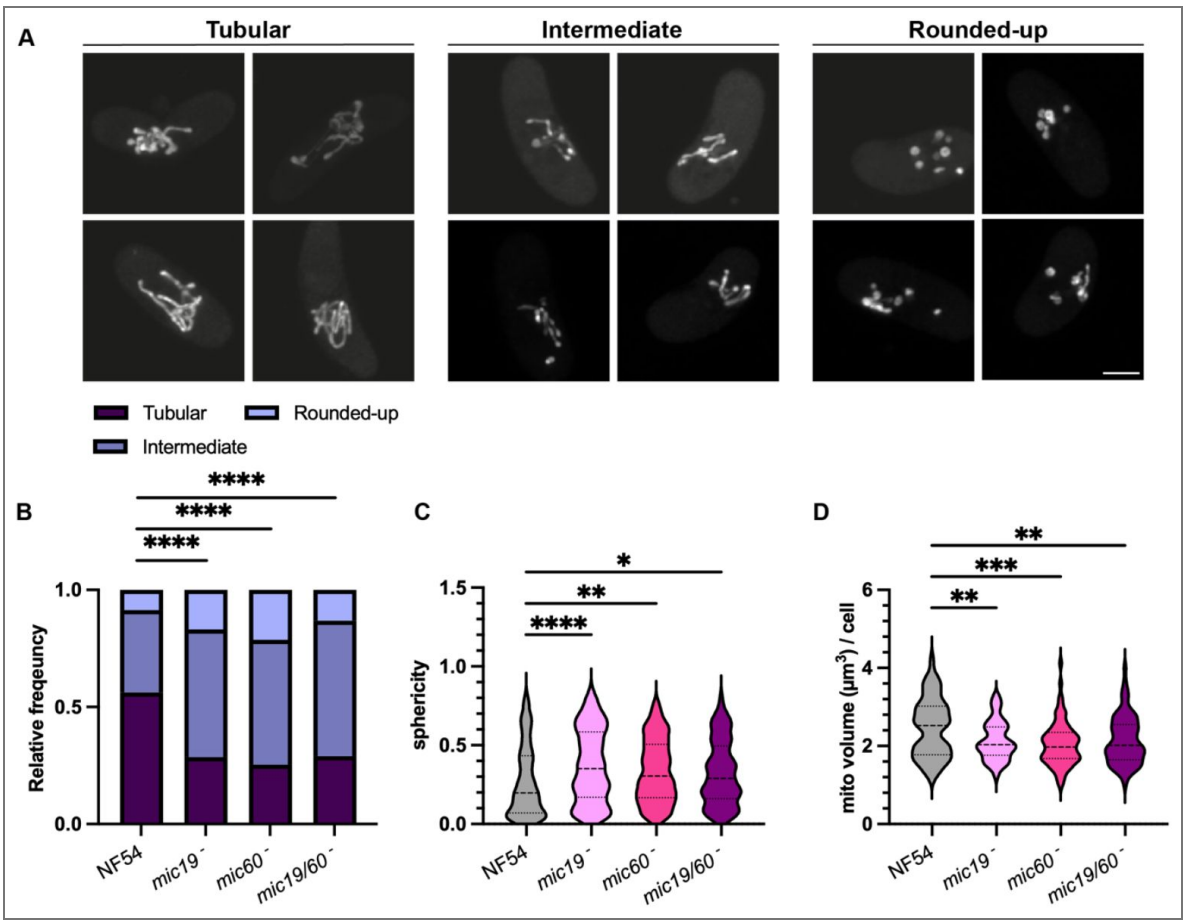

Figure 3. Fluorescence microscopy reveals aberrant mitochondrial morphology in mature P. falciparum mic19⁻, mic60⁻, and mic19/60⁻ gametocytes.

A) Representative fluorescent microscopy images of mature NF54 gametocytes with tubular, intermediate, or rounded-up mitochondria. Samples were stained with MitoTracker. Images are maximum intensity projections of Z-stack confocal Airyscan images. Scale bars: 2 µm. B) Analysis of mitochondrial shape based on mean branch length per cell. Rounded-up = branch length < 2 µm, intermediate = branch length between 2 µm and 10 µm, tubular = branch length > 10 µm. Statistical analysis using Chi-squared test with Bonferroni correction for full comparison between all cell lines; significance is indicated as follows: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. C) sphericity of mitochondria, D) mitochondrial volume per cell. In all cases n° of replicates > 3 with total number of cells ≥ 75. Statistical significance calculated with one-way ANOVA with Sidàk correction for full comparison between all cell lines; significance is indicated as follows: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

diameter of cristae, as the data is two-dimensional. The recorded values are to be considered indicative of trends, rather than absolute dimensions of cristae. Even though cristae of the two single knockout lines (*mic19*$^{-}$ and *mic60*$^{-}$) appeared disorganized, significant increase in apparent length (but not diameter) of cristae was only detected in *mic19/60*$^{-}$ gametocytes (with a mean crista length of 155 nm compared to 96 nm for NF54) (Figure 4B-C). Despite the lack of overall significance of these changes in the single knockouts, the spread of recorded crista length did increase in all knockout lines, therefore indicating a trend towards heterogeneity of crista architecture. In line with those observations, crista area increased in all knockout lines (Figure 4D). We expect this effect to translate into the third dimension and thus conclude that the mean crista volume increases with the loss of either *PfMIC19*, *PfMIC60*, or both. Even though the deletion of *PfMIC19* and/or *PfMIC60* clearly affected crista morphology, mean cristae coverage per mitochondrial cross-section did not significantly change in *mic19*$^{-}$ and *mic60*$^{-}$ (Figure 4E). The double knockout line (*mic19/60*$^{-}$), however, reached significance due to a small population of severely aberrant cristae (Figure 4F and Figure S7). We observed instances of extremely long cristae, as well as mitochondria with large single- or double-membraned circles within the mitochondrial cross-section. The reason for the lack of a significant change in *mic19*$^{-}$ and *mic60*$^{-}$ despite the increased area per crista can be calculated from the same datapoints and shows that the mean number of crista cross-sections per μm^2 of mitochondrial cross-section drops from 33.8 in NF54 to 20.3, 16.3 and 26.4 in *mic19*$^{-}$, *mic60*$^{-}$ and *mic19/60*$^{-}$, respectively. These effects in combination suggest that the increased area per crista is associated with a reduced number of cristae per mitochondrial area. Given the observed aberrations in crista morphology in knockout lines, we conclude that membrane invaginations can still form, even in the absence of *Pf*MIC19 and *Pf*MIC60, suggesting that these proteins are not essential for initiating crista formation. Nevertheless, *Pf*MIC60 and *Pf*MIC19 both play an essential role in shaping nascent cristae. Furthermore, our data indicates the effect size increases with simultaneous ablation of both proteins.

*Pf*MIC60 and *Pf*MIC19 are important for proper crista junction formation

To delve deeper into the effects of *PfMIC19* and *PfMIC60* deletions, we performed dual-tilt TEM tomography on 250 nm sections of the same samples used in Figure 4. The phenotypes matched our observations from two-dimensional acquisitions. We found crista morphology to be altered in all knockout lines, with most severe aberrations found in *mic19/60*$^{-}$ (Figure 5A-B). We manually segmented one representative mitochondrion per parasite line to visualize the observed effects (Figure 5C and Movies 1-4). We observed that in all knockout parasites, we rarely identified connections between the crista membranes and the boundary membrane. To quantify the observed loss of membrane connectivity, we analysed the connectivity of all cristae within mitochondrial volumes from different gametocytes (Figure 5E-F). We thoroughly searched for crista junctions of each crista found within the mitochondrial segments. Note that these mitochondrial volumes are not full mitochondria, but large segments thereof. As a result of the incompleteness of the mitochondria within the section, and the tomography specific artefact of the missing wedge, we were unable to confirm whether cristae were in fact fully detached from the boundary membrane, or just too long to fit within the observable z-range. Instead, we quantified the fraction of cristae for which we identified a crista junction (Figure 5E). While we identified crista junctions for 55% of the cristae seen in NF54 gametocytes, we could only confirm crista junction presence 9% of the cristae traced in *mic19*$^{-}$ and 23% of the cristae in the *mic60*$^{-}$ sample. Similarly, connectivity also dropped in the double knockout, with 21% of confirmed crista junctions. Note that for *mic19/60*$^{-}$ we chose to measure connectivity in mitochondria that we considered representative for the population average rather than the extreme cases shown in (Figure 4F and Figure S7). For each parasite line, we measured the diameter of at least 12 crista junctions (Figure 5E-F). A noteworthy, yet non-significant drop of the crista junction diameter was observed in *mic19/60*$^{-}$, where the mean crista junction diameter dropped from 25

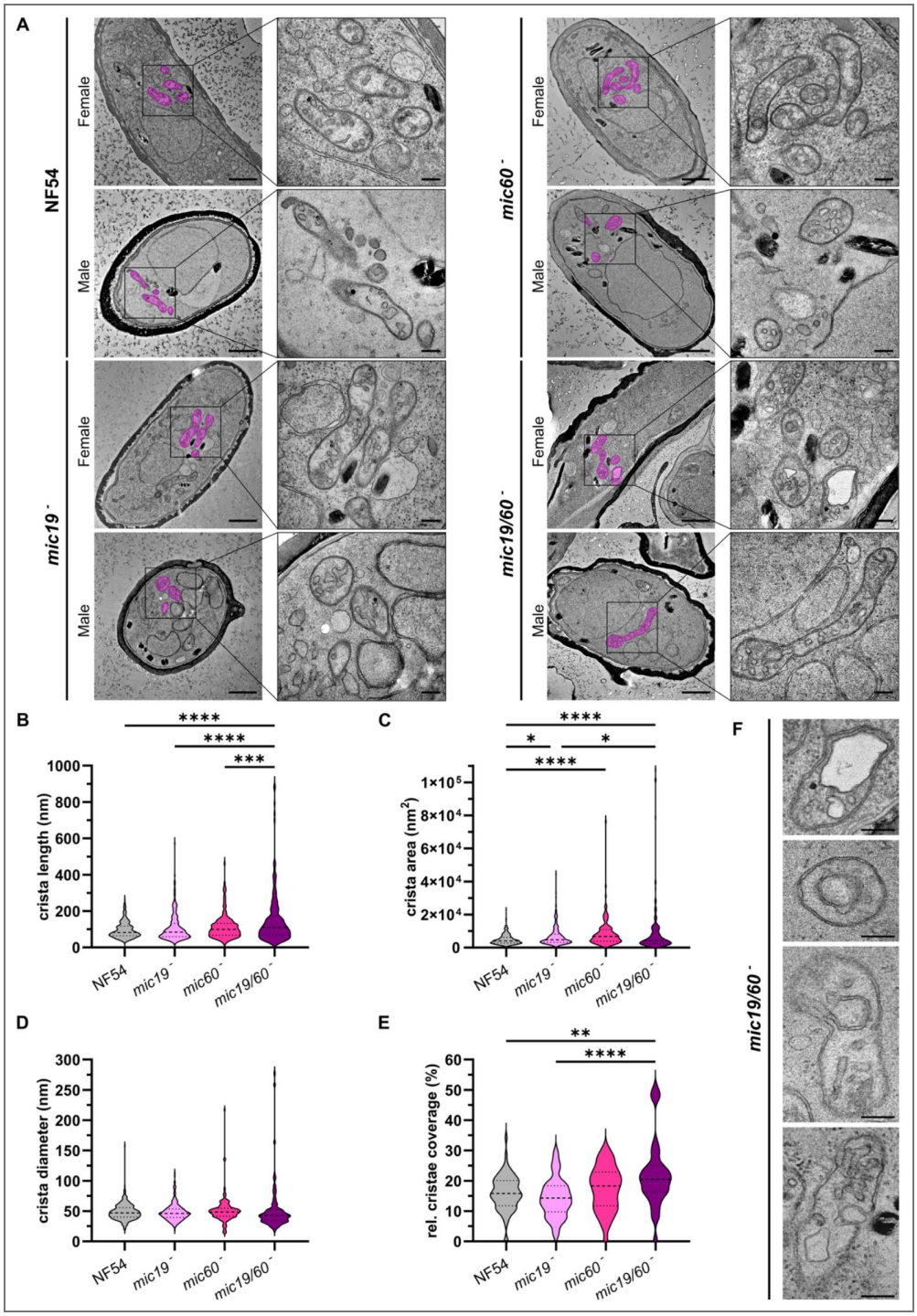

Figure 4. Transmission electron microscopy reveals aberrant crista structures in mature P. falciparum mic19⁻, mic60⁻, and mic19/60⁻ gametocytes.

A) Representative micrographs of NF54 (WT), mic19⁻, mic60⁻, and mic19/60⁻ gametocytes. Mitochondria within a rectangular selection are highlighted in pink. Zoom-ins on these selections are shown on the right side of each micrograph. For each cell line, a male and a female parasite are shown. Scale bars: 1 µm / 200 nm. B - D) Quantification of the length (log) (B), width (log) (C), and area (log) (D) of cross-sections through individual cristae (n ≥ 100, from ≥ 30 mitochondria). E) Quantification of cristae coverage based on the total crista area of a mitochondrial cross section relative to the area of the respective mitochondrial cross section (n ≥ 30). F) Examples of severely aberrant mitochondria as observed in TEM of mic19/60⁻ gametocytes. Scale bars: 200 nm. Statistical analysis of B – E: one-way ANOVA with Sidàk correction for full comparison between all cell lines; significance is indicated as follows: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

nm in the NF54 control to 20 nm in *mic19/60*$^{-}$. Collectively, these results demonstrate that the deletion of *PfMIC19* and/or *PfMIC60* leads to a reduced number of crista junctions and a possible reduction in crista junction diameter in *mic19/60*$^{-}$.

Discussion

MICOS is a protein complex of the inner mitochondrial membrane that is involved in the organization of mitochondrial cristae and stabilization of crista junctions (3, 8-10). Though well established as the cristae organizing system in opisthokonts (the clade including animals and fungi), MICOS has not previously been studied in apicomplexan parasites (38). The human malaria parasite *P. falciparum*, offers a novel perspective on cristae biology, as it is one of very few species that reportedly grow cristae *de novo* from an acristate mitochondrion (25, 39, 40). Due to its medical relevance and the central role of the mitochondria as a validated drug target (41), *P. falciparum* is an invaluable model to study the role of MICOS in cristae organization and formation. Recent bioinformatic data revealed a putative *Pf*MIC60, thus priming the investigation of other components of the complex (14, 23). In this study, we investigated the roles of the putative *PfMIC60* and *PfMIC19* orthologues in the formation and organization of cristae.

The *P. falciparum* mitochondrial proteome intensely varies between different life cycle stages. In fact, almost all proteins involved in oxidative phosphorylation are dramatically increased in gametocyte stages compared to ABS (40, 42). The same trend was observed for *Pf*MIC19 and *Pf*MIC60. Although transcriptomic data suggested that low-level transcripts of *PfMIC19* and *PfMIC60* might be present in ABS (43, 44), we did not detect either of the proteins in ABS by western blot analysis. The apparent absence could indicate that transcripts are not translated in ABS or that the proteins' expression was below detection limits of western blot analysis. In contrast, both proteins were detectable, albeit in low abundance, in mature gametocytes. The expression patterns of *Pf*MIC19 and *Pf*MIC60 are consistent with the absence of cristae in ABS and their appearance during gametocytogenesis (25, 40). The low abundance of *Pf*MICOS components even in mature gametocytes likely contributed to our difficulties confirming mitochondrial localization of *Pf*MIC19 and *Pf*MIC60 using fluorescence microscopy.

The apparent absence of *Pf*MIC19 and *Pf*MIC60 in ABS also explains the lack of a growth defect in this part of the life cycle. Surprisingly though, gametocytes and mosquito stages, that are believed to strongly rely on oxidative phosphorylation for their survival (40, 42), were largely unaffected by depletion of these MICOS components, with the exception of a slight reduction in mosquito colonization. This lack of growth arrest has also been described for the knockout of some MICOS components in human cells, yeast and *Trypanosoma brucei* (16, 45-47). Though, in some cases, MICOS subunits were shown to be essential for cell survival and growth, such as the cryptic mitofilin MIC34 and MIC40 in *T. brucei* (48). In addition, disruption of some MICOS subunits has been reported to partially impair cell respiration (10, 20, 49, 50). Considering these relationships, successful propagation of mosquito stages suggests either that the growth defect is masked by the unnaturally rich environment encountered in blood culture medium (51) or that the reported impact on respiratory complexes does not occur to a relevant extent in *P. falciparum* which is supported by the absence of detectable differences in membrane potential .

Consistent with prior observations in human cells (10, 52), knockouts of *Pf*MIC19 and *Pf*MIC60 strongly altered mitochondrial morphology. In fact, both single and the double knockout parasite lines exhibited mitochondria that were more rounded-up and spherical. The presence of rounded-up mitochondria upon knockout of MIC60 and MIC19 has also been observed in human and yeast cells and appointed as a physiological response to mitochondrial stress (10, 20). Given this background and the extreme variability in mitochondrial shape we assume mitochondrial rounding-up in *P. falciparum* is also a consequence of mitochondrial stress, due to the loss of *Pf*MIC19 and *Pf*MIC60, rather than a direct involvement of these proteins in mitochondrial shape. Our observation of this mitochondrial rounding-up in the NF54 parental control, could indicate a baseline level of mitochondrial stress in culture conditions. Similarly, we cannot clearly appoint the reduction in oocyst number to a direct response to the loss of *Pf*MIC19 and/or *Pf*MIC60, as it may reflect mitochondrial stress or even result from the high variability enclosed in the assay

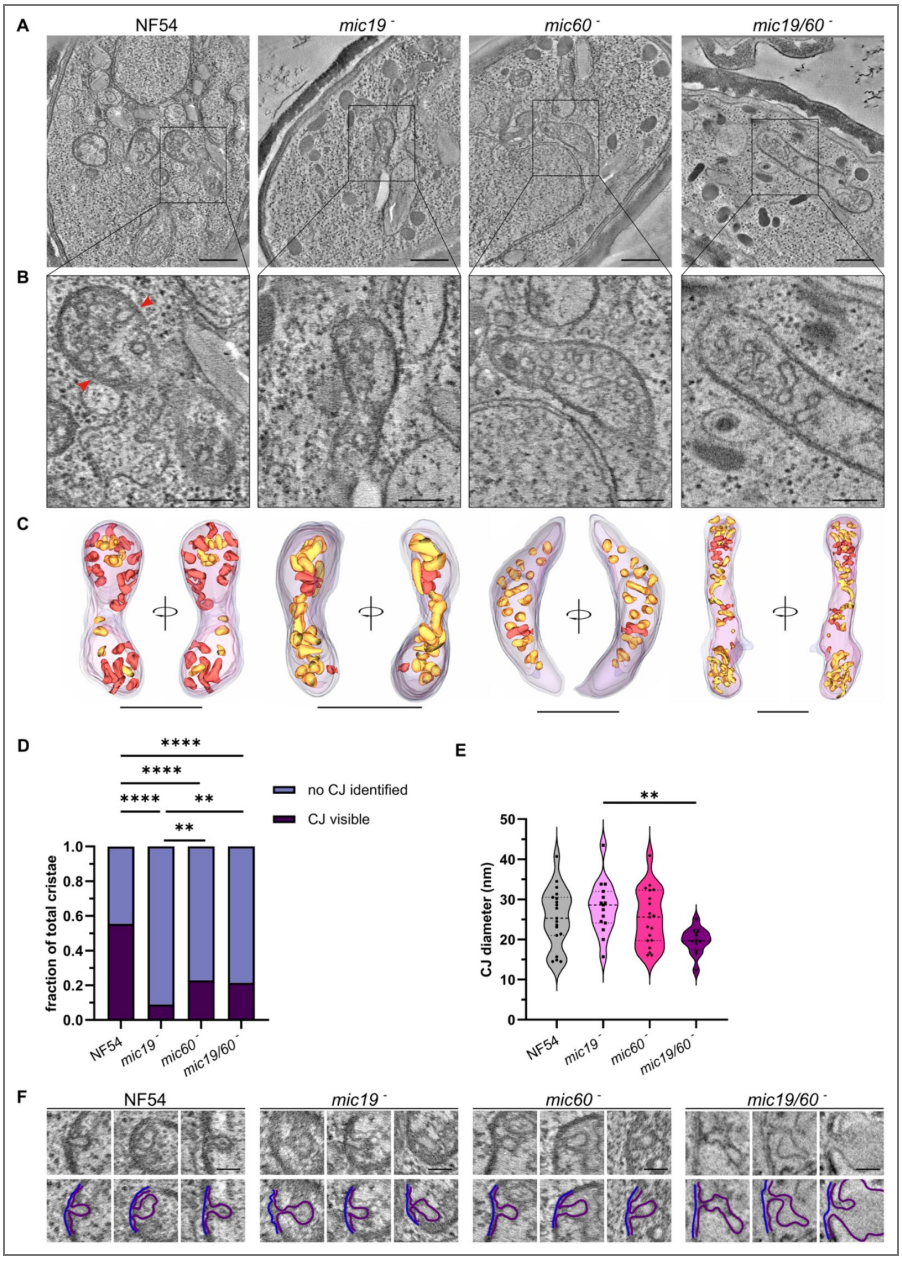

Figure 5. Transmission electron tomography reveals a drop in crista junction prevalence in mature P. falciparum mic19⁻, mic60⁻, and mic19/60⁻ gametocytes.

A) Representative snapshots through TEM tomograms of mature NF54 (WT), mic19⁻, mic60⁻, and mic19/mic60⁻ gametocytes. Scale bars: 500 nm. B) Zoom-ins of individual mitochondria from the tomograms shown in A. Crista junctions highlighted with red arrow heads. Scale bars: 200 nm. C) 3D model of 250 nm mitochondrial sections of the mitochondria shown in B. Each model is shown from two angles 180° to each other. Scale bars: 500 nm (full tomograms and mitochondrial segmentations in Movies 1-4). For red coloured cristae, a CJ was identified within the inspected 250 nm section of the mitochondrion. For yellow-coloured cristae we could not identify connection to the boundary membrane within the tomogram. D) Quantification of the relative proportions of cristae for which a crista junction (CJ) was observed within the 250 nm section (n ≥ 56). Statistical analysis using Chi-squared test with Bonferroni correction for full comparison between all cell lines; significance is indicated as follows: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. E) Quantification of the diameter of crista junctions at their narrowest point (n ≥ 12, from several mitochondria per sample). Statistical analysis using one-way ANOVA with Sidàk correction for full comparison between all cell lines; significance is indicated as follows: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. F) Three representative crista junctions from mature NF54, mic19⁻, mic60⁻, and mic19/mic60⁻ gametocytes. Scale bars: 100 nm. 2D segmentation of the outer mitochondrial membrane (blue) and the inner mitochondrial membrane (violet) shown in the bottom row.

itself. Still, it is striking that, despite the pronounced morphological phenotype, and the possibly high mitochondrial stress levels, the parasites appeared mostly unaffected in life cycle propagation, raising questions about the functional relevance of crista architecture and overall mitochondrial morphology at these stages.

The MitochondriaAnalyzer plug-in for FIJI, used here for the first time on *P. falciparum* samples, proved to be a potent tool to improve bulk quantification of fluorescence microscopy data (36). Of course, it is also important to consider that while fluorescent microscopy is a powerful technique, it cannot reliably determine the number of mitochondria in *P. falciparum* gametocytes. As shown by TEM data, mitochondria are often located in very close proximity (25, 53), thus the fluorescence-based mitochondrial counts reflect more on whether mitochondria are sufficiently dispersed to be recognized as separate, rather than providing an absolute count.

The depletion of either or both *P. falciparum* MICOS genes leads to alterations in the organization of cristae membranes while total cristae coverage remains largely unaffected, thus suggesting that membrane invaginations still arise but are not properly arranged in these knockout lines. While the extent of cristae disruptions in *mic19*$^{-}$ and *mic60*$^{-}$ gametocytes was comparable (as shown in thin section TEM and TEM tomography), the double knockout parasites showed stronger morphological defects compared to the single knockouts. This may suggest that in *P. falciparum* the depletion of just one of these proteins does not fully disrupt MICOS function, Instead, our data indicates progressive loss of MICOS function upon the depletion of multiple MICOS components.

While MIC60 knockout in human cells showed an almost complete loss of crista junctions (10), cristae aberrations were more subtle in our knockout lines. Nevertheless, we did see a decrease in crista junction abundance in all lines, and a trend that is indicative of a reduction of crista junction diameter in the double knockout. Interestingly, in both human and yeast cells, crista junctions exhibit diameters ranging from 12 to 40 nm, with an average of 28 nm, which is consistent and comparable with our observation in the parental NF54 gametocytes (54-56). The change in crista junction diameter suggests that, at least in *mic19/60*$^{-}$, the remaining crista junctions are affected by the loss of *Pf*MIC60 and *Pf*MIC19. It remains uncertain, though possible, that protein-mediated stabilization is entirely absent in the double knockout.

It is important to note that effects on crista junction structure upon destabilization of the MIC60 subcomplex is a phenomenon described also in yeast and human cells. (10, 19). Previous studies showed that depletion of *MIC60* in HeLa cells led to an increase in crista junction diameter, while the depletion of *MIC19* did not affect this parameter (11). In contrast to that, destabilization of the MIC60 tetramer in yeast led to a decrease in crista junction diameter (19). Interestingly, our findings do not suggest effects on crista junction diameter for either one of the single knockouts, while the double knockout resulted in a 20% reduction. The central, tetrameric coiled coil domain of MIC60 has been suggested to define the diameter of the crista junction (19). While not experimentally proven, MICOS induced stabilization of crista junction diameter is a plausible mechanism also for *P. falciparum*. In fact, the mean diameter of *P. falciparum* crista junctions aligns well with the theoretical length of the coiled coil domain predicted by AlphaFold.

Collectively, we have demonstrated that *Pf*MIC19 and *Pf*MIC60 are putative mitochondrial proteins expressed in gametocytes. Their deletion does not affect ABS nor sexual blood stages in standard culture conditions. While oocyst counts were modestly reduced, sporozoite production did not appear to be affected, indicating that the proteins are not essential at any point of the parasite life cycle investigated in this study. Still, knockout of *Pf*MIC19 and *Pf*MIC60 strongly altered mitochondrial morphology in gametocytes. Mitochondrial cristae are aberrant but present, with a reduction in crista junction abundance, confirming a role in *Pf*MICOS. Although molecular mechanisms of membrane bending and anchoring are yet to be elucidated, our study demonstrated the existence of *Pf*MICOS. In contrast to other systems where MICOS has been described, *P. falciparum* further allows us to clearly decipher cause-effect relationships in this context. We conclude that even in the absence of *Pf*MIC60 and *Pf*MIC19, cristae are formed *de novo* and allowing for parasite survival. Thus, our data provide direct evidence that components of *Pf*MICOS are essential for crista junction organization and stabilization, but not for initial cristae formation. This functional separation is likely to apply beyond *P. falciparum*, to evolutionary

distant organisms such as human and yeast (22). Our results highlight the unique utility that the two-state mitochondrial architecture in *P. falciparum* provides to the field of mitochondrial biology and lays the groundwork for future studies on the causal dissection of crista biogenesis during *P. falciparum* gametocytogenesis and beyond.

Material and Methods

Plasmid generation

To generate the *mic19-HA* and *mic60-HA* homology-direct repair (HDR) plasmids, the pRF0139 empty tagging plasmid was used, containing 3xHA-GlmS, PBANKA_142660 bidirecoonal 3'UTR, and the mitochondrial-mScarlet marker (31). 5' and 3' homology arms (HRs) were amplified from genomic NF54 DNA and cloned with specific restricoon enzymes (Table S1).

HDR templates for *mic19*$^{-}$ and *mic60*$^{-}$ were generated from pGK backbone (57), which contains a pBAT backbone (58) with H2B promotor, GFP and PBANKA_142660 bidirecoonal 3'UTR. The 5' and 3' HRs, amplified from NF54 gDNA, were cloned into pGK using specific restricoon sites (Table S1).

To generate the HDR template for the *mic19/60*$^{-}$, 5' and 3' HR were inserted into pCK2a, which contains a pBAT backbone (58) with H2B promotor, mCherry and PBANKA_142660 bidirecoonal 3'UTR.

Cas9 guide plasmids were generated inserong a pair of annealed complementary, guide encoding, oligonucleoodes into pBF-gC (59) (kind giq from Till Voss) or, pRF0568, a plasmid derived from pMLB626 (60) (kind giq from Marcus Lee) without a 3xHA tag fused to *Cas9*. BsaI and BbsI, respecovely, restricoon enzymes were used to insert the oligonucleoodes and generate the final guide plasmids (Table S1). All PCR reacoons were performed using Primestar GXL DNA polymerase (Takara Bio), and all cloning enzymes were provided by New England Biolabs.

P. falciparum culturing and transfection

P. falciparum ABS were cultured in RPMI medium (with 25 mM HEPES, 100 mM hypoxanthine, and 24 mM sodium bicarbonate) completed with 10% AB+ human serum and 5% 0+ human Red Blood Cells (RBCs) (Sanquin, The Netherlands) (61). The cultures were kept at 37° C in a low oxygen environment (3% O_2, 4% CO_2). When needed, ring-stage parasites synchronization was achieved using sorbitol treatment (62). Culture parasitaemia, was monitored with Giemsa-stained blood smears using a 100X oil objective.

For transfections 60 µg of each Cas9 guides and linearized repair plasmids were ethanol precipitated and transfected on a ring-stage synchronized culture, as previously described (63). Briefly, the plasmids were dissolved and mixed with cytomix (0.895% KCl, 0.0017% $CaCl_2$, 0.076% EGTA, 0.102% $MgCl_2$, 0.0871% K_2HPO_4, 0.068% KH_2PO_4, 0.708% HEPES) for a total volume of 300 µl. The DNA was then added to 0.4 ml of packed ring-stage infected RBCs, and the solution was electroporated (0.31 kV and 950 µF). Parasites were left to recover for 4 h after transfection in complete medium and after that treated in 2.5 nM WR99210 (Jacobus Pharmaceuticals) or 40 µg/ml blasticidin (Gibco #R210-01) for 5 days. To obtain an isogenic population, parasites were isolated using flow cytometry (64) based on GFP (*mic19*$^{-}$ and *mic60*$^{-}$), mCherry (*mic19/60*$^{-}$) or mScarlet (*mic19-HA* and *mic60-HA*) expression, using a CytoFLEX SRT benchtop Cell Sorter (Beckman Coulter). Transfected parasites were cultured until recovered and integration was confirmed by diagnostic PCR (Table S1).

Growth assay

Parasite cultures were tightly synchronized through a double sorbitol synchronisation performed at 0 h and 16 h. Ring-stage parasites were determined through flow cytometry and cultures were diluted to have a 0.5% final parasitaemia. Cultures were set up at 0.5% parasitaemia and samples were harvested for 4 consecutive days. Samples for flow cytometry were taken directly after set-up, on day 1, 2 (before and after dilution), 3 and 4. On day 2 all cultures were diluted with the same dilution factor (1/20). When harvested, samples were fixed in 0.25% glutaraldehyde (ITW

Reagents, CA0589.100) in PBS and, before flow cytometry (CytoFLEX - 13-color, Beckman Coulter), stained with SYBR™ green (ThermoFisher, 7563) 1:10.000 for 30 min at 37° C. Data was analysed using FlowJo software (version 10.10).

Gametocyte culture conditions and standard membrane feeding assay

Sexual conversion in parasites was induced in a trophozoite-stage culture, adding Albumax-containing medium for 36 h and changed every 12 h (65). Gametocytes were then cultured with normal culture medium until maturation. During the entire procedure gametocytes were kept at 37° C in low oxygen conditions. To remove ABS from the culture, gametocytes were cultured in the presence of heparin (20 U/ml) for 4 days. To quantify exflagellation, cultures were mixed 1:1 with 50 µM xanthurenic acid in complete RPMI medium. The solution, kept at room temperature for 15 min, was then transferred to a Neubauer chamber where the exflagellation centres were counted with a 40X objective, bright-field microscopy. To assess mosquito infectivity, day 12 mature gametocytes (65) were added to the bloodmeal of *Anopheles stephensi* mosquitoes (colony maintained at Radboudumc, Nijmegen, The Netherlands), as previously described (66). Mean oocysts per midgut and statistical significance were calculated using a generalized linear mixed effect model with a random experiment effect under a negative binomial distribution. The fitted model was used to obtain estimated means and contrasts that were evaluated using Wald statistics. All datasets, statistical analysis and p-values are reported in Supplementary Information 2.

Fluorescence microscopy

Mature gametocytes were incubated in 100 nM MitoTracker (MitoTracker® Orange CMTMRos, Thermo Fisher, #M7510) diluted in RPMI medium for exactly 30 min at 37° C and afterwards washed and diluted 1:10 in incomplete medium. The samples were allowed to settle on poly-L-Lysin coated coverslips (Corning, #354085) for 30 min at 37° C before being fixed in 4% paraformaldehyde (Thermo Fisher, #28906) and 0.0075% glutaraldehyde (Panreac, #A0589,0010) in PBS for 20 min (67). The samples were then washed with 1X PBS and permeabilized with 0.1% Triton X-100 in PBS for 10 min. Cells were blocked with 3% BSA for 1 h at RT. The coverslips were incubated with primary antibodies diluted in PBS with 3% BSA (Table S2) for 1 h at RT, washed three times and then incubated with the secondary antibodies for 1 h at RT. All samples were additionally stained with 300 nM DAPI (Thermo Fisher, #62248) for 1 h before being mounted onto microscope slides with Fluoromount G (Thermo Fisher, # 00-4958-02). Images were acquired using an LSM900 confocal microscope with airyscan (ZEISS) using a 63x oil objective with 405, 488, 561, and 633 nm laser excitation and an Electronically Switchable Illumination and Detection Model (ESID) for transmitted light. Images belonging to the same experiment were acquired with equal settings for laser power and detector sensibility and afterwards analyzed with the same parameters to compare expression.

Mitochondria quantification in fluorescence microscopy

Mature gametocytes prepared as previously explained, were randomly selected based on DIC or DAPI signal to avoid bias. Mitochondria were quantified using the Fiji plug-in MitochondriaAnalyser (36). Briefly, 3D image stacks were pre-processed using the following commands: subtract background (radius = 1.25 µm); sigma filter plus (radius = 2.0 µm); enhance local contrast (slope = 1.40 µm); and gamma correction (value = 0.9 µm). For thresholding, block size was set to 0.85 µm with a C value of 7. Thresholded images were then postprocessed using the functions despeckle and remove outliers with radius = 3 pixels. Statistical significance was determined using one-way ANOVA test with Sidàk correction. Mitochondria were divided in tubular, intermediate and round up based on average branch length (total branch length / number of mitochondria intensities) and statistical significance was determined using a Chi-squared test. To assess membrane potential the same samples were used to quantify MitoTracker signal. The

automatic tresholded images were used to determine background-corrected mean fluorescence intensity in the z-stack. All datasets, statistical analysis and p-values are reported in Supplementary Information 2.

Ultra-Expansion Microscopy (U-ExM)

Mature gametocytes were isolated using MACS LS columns (Miltenyi Biotec). Shortly, LS columns were equilibrated using incomplete RPMI medium and mature gametocyte cultures were flushed in the LS columns attached to a magnet using a 23G needle to regulate the flow. To elute gametocytes, columns were removed from the magnet and washed with incomplete medium. MACS column, magnet and incomplete RPMI medium were previously incubated at 37° C, and kept at this temperature throughout the procedure, to prevent gametocyte activation during purification. The gametocyte pellet was then incubated with 100 nM MitoTracker and fixed as described for fluorescence microscopy. Coverslips were then incubated with 0.5% acrylamide (AA) (Sigma, #A4058) and 0.38% formaldehyde (FA) (Sigma, #F8775) in PBS overnight at 37° C. Gelation was performed as previously described (68). Briefly, the gelling solution was prepared by adding tetramethylethylenediamine (TMED, final concentration 0.5%) (Sigma, #T7024) and ammonium persulfate (APS, final concentration 0.5%) (Sigma, #A3678) to U-ExM monomer solution (MS) (23% w/v sodium acrylate (AK Scientific, #R624), 10% w/v AA, N,N'-methylenbisacrylamide (BIS) (Sigma, #M1533) 0.1% w/v in PBS). The solution was then added to the coverslips and incubated at 37° C for 1 h. Gels were incubated with denaturation buffer (200 mM SDS, 200 mM NaCl, 50 mM Tris in water, pH 9) under strong agitation until complete detachment from the coverslips and transferred into a 1.5 ml Eppendorf tube filled with denaturation buffer and incubated 90 minutes at 95° C. Gels were expanded overnight in ultrapure water at RT. Gels were then incubated with primary antibodies (Table S2) for 3 h at 37° C while shaking, followed by secondary antibodies, NHS-ester (8 µg/ml) (DyLightTM 405 NHS-ester, ThermoScientific, #46400) and SYTOX™ (Thermo Fisher, #S11381) for 2.5 h at 37° C, shaking. For imaging, gels were cut into pieces and positioned on a poly-D lysine (Gibco, #A38904-01) coated coverslips. Images were acquired with identical parameters, using an LSM900 confocal microscope with airyscan (ZEISS) with a 63X oil objective at 405, 488, 561, and 633 nm laser excitation and an Electronically Switchable Illumination and Detection Model (ESID) for transmitted light.

Electron microscopy sample preparation

Mature gametocytes were harvested using MACS LS columns as described for U-ExM. Purified gametocytes were then fixed with 4% PFA in 0.1 M PHEM and pelleted at 600 x g for 10 min. Pellets were vitrified using the Leica EMPACT high pressure freezer with membrane carrier assembly. Water removal and staining was achieved by freeze substitution in 1% osmium tetroxide, 0.2% uranyl acetate and 1% water in acetone. Stepwise freeze substitution consisted of a 48 h incubation at -90° C, sloped increase to -60° C within 12 h, 10 h incubation at -60° C, sloped increase to -60° C within 12 h, incubation at - 30° C for 8 h and removal of uranyl acetate by washing in 1% osmium tetroxide in acetone at -30° C. The samples were then transferred to ice, osmium tetroxide was replaced with pure acetone, and the samples were further stained with 1% tannic acid in acetone for 1 h and with 1% osmium tetroxide in acetone for 1 h. Then the samples were embedded in Epon (46% HY 964 Araldite, 39.6% Epoxy Embedding Medium, 13% MNA, 1.4% DMP-30) in a stepwise increase of the Epon to acetone ratio. Fully infiltrated samples were hardened at 70° C for 5 days. Thin sections of 65 nm or semi-thin sections of 250 nm were collected on copper grids with formvar/carbon coating. Thin sections were post stained with 2% uranyl acetate for 5 min and Reynolds lead citrate (Reynolds, 1963) for 2 min. Semi-thin sections were labelled with 15 nm gold fiducials for tilt-series alignment.

Transmission electron tomography

All tomograms were recorded at a JEOL2100 TEM operating at 200 kV. Tomograms were recorded as dual-axis tilt series at 15k magnification, 1.5° tilt increment ranging from -60° to 60° (pixel size 1.52 nm, defocus –1.1 µm). Reconstruction, segmentation and quantifications were performed

manually, in IMOD. The statistical significance of the deviations in crista junction diameter was determined using one-way ANOVA test with Sidàk correction. Significance of the difference in crista junction prevalence was tested using a Chi-squared test. All datasets, statistical analysis and p-values are reported in Supplementary Information 2.

Thin section electron microscopy

Micrographs were collected at a JEOL1400 TEM operating at 120 kV. Recorded images were randomized for blinded quantification of crista length, diameter, area, and coverage. Statistical significance was determined using one-way ANOVA tests with Sidàk correction. All datasets, statistical analysis and p-values are reported in Supplementary Information 2.

SDS-Page gel and western blot

Saponin pellets of gametocytes and ABS cultures were prepared by resuspending in 7X pellet volume ultrapure water and 2X pellet volume reductive loading buffer (1% β-mercaptoethanol, 0.004% bromophenol blue, 6% glycerol, 2% SDS 50 mM Tris-HCl, pH 6.8). Samples were heated to 70° C for 10 min, quickly vortexed and spun down to precipitate insoluble debris and DNA. The supernatant was loaded on a SurePAGE, 8% Bis-Tris SDS-PAGE (GenScript, #M00677). A standard protein ladder (Precision Plus Protein in Dual Colour Standard, Bio-Rad) was included in every run. Proteins were transferred, with the semi-wet transfer, to a nitrocellulose membrane soaked in transfer buffer (20% methanol with 10% Tris-Glycine (10X)) in a Trans-Blot Turbo machine (Bio-Rad) at 25V for 30 min. The membrane was then blocked in 1% milk powder in PBS with 0.05% Tween 20 (PBST) overnight at 4° C. Primary and secondary antibodies (Table S2) were diluted in 1% milk powder in PBST and incubated for 1 h at RT. The blot was then imaged with Odyssey CLx (ImageQuant).

Structural comparisons and domain predictions

All AlphaFold models were retrieved from the AlphaFold protein structure database (ScMIC60: AF-P36112-F1-v4, *Pf*MIC60: AF-. Q8IJW5-F1-v4, ScMIC19: AF-P43594-F1-v4, *Pf*MIC19: AF-Q8IIR5-F1-v4) (32, 33). Domains were used as predicted by TED consensus domain, as integrated in the AlphaFold protein structure database (34). Associated pLDDT prediction confidence information is shown in Figure S1. Theoretical length of the coiled coil domain in ScMIC60 and *Pf*MIC60 was measured by atomic distance within the predicted models.

Data availability

All TEM tomography datasets are available in the Electron Microscopy Data Bank (EMDB) under accession codes EMD-54915 (NF54), EMD-55186 (*mic19*$^{-}$), EMD-55149 (*mic60*$^{-}$) and EMD-55336 (*mic19/mic60*$^{-}$).

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Additional information

Author contributions

LvN and TWAK obtained funding and conceptualized and supervised the project; STL, IB and JLO performed experiments; STL, IB, JLO, FE, TWAK and LvN analysed data; NIP, GJvG and RS executed the mosquito experiments; STL and IB wrote the first draft of the manuscript and all authors reviewed, edited and approved the manuscript.

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Additional files

Movie 1

Movie 2

Movie 3

Movie 4

Supplementary Information 1

Supplementary Information 2

Supplementary Figures

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Peer reviews

Reviewer #1 (Public review):

The manuscript by Tassan-Lugrezin et al. confirms the existence of the MICOS complex in the causative agent of malaria Plasmodium falciparum. Prior to this study, only one of the two core MICOS subunits, Mic60, was found by homology search to be encoded in the apicomplexan parasite's genome. This study demonstrates the absence of the other core subunit, Mic10. It also identifies another MICOS subunit, Mic19, which co-migrates with Mic60 in a very large molecular weight complex upon blue native polyacrylamide gel electrophoresis. The authors then demonstrate that expression of both Mic60 and Mic19 is considerably upregulated during the differentiation of P. falciparum from the pathogenic asexual blood stage (ABS) to gametocytes, which correlates with the activation of oxidative phosphorylation during this process. While gene deletion of Mic19, Mic60 and both simultaneously does not affect this transition, the crista are nevertheless deformed. More significantly, crista junctions are significantly reduced, indicating that MICOS serves the same function in apicomplexans as it does in opisthokonts: maintaining crista junctions. Furthermore, the genetic interaction of mic60 and mic19 observed by augmented crista deformation when both are deleted is further evidence of their biochemical interaction, further supporting their similar complexome profiles. This study represents an important contribution to our understanding of MICOS evolution. Furthermore, the study shows that proper cristae formation is not essential for Plasmodium life cycle progression under in vitro conditions. Moreover, mutant gametocytes are still able to mate in the mosquito vector, albeit with lower efficiency.

Strengths:

The study is a result of a lot of technically challenging work in the model Plamsodium. The technically difficult life cycle progression experiments are well performed as far as I can tell. The electron microscopy is very well done and rigorously analyzed to obtain information about crista parameters. In particular, the authors were able to quantify the occurrence and diameter of crista junctions, which is very challenging in small mitochondria with small cristae. Finally, the authors provide convincing support that Mic60 and the newly discovered Mic19 act to shape crista junctions and MICOS can apparently carry out this function without the core subunit Mic10.

Weaknesses:

In its current form, there are conceptual weaknesses. The authors focus on the development of crista from a highly likely acristate state. This is true. But there can be more insight by considering their result in light of discovering the first functioning MICOS complex without one of its two core proteins, Mic10. The surprisingly large size of is also not really considered by the authors. This brings me the second weakness in my opinion. While I think the study represents a lot of work utilizing appropriate and crucial experiments, it seems the Complexome data was not explored enough. This data revealed Mic19, but what other potential subunits are co-migrating with Mic60 and Mic19 that can explain the large size of Plasmodium MICOS? Also, what is the consequence of the loss of Mic60 and Mic19 on the mitoproteome? Perhaps other MICOS subunits can be identified by their depletion in the knockouts versus the parental cell line.

Comments on latest version:

I am reviewing this manuscript again after reviewing it for Reviewers Commons. I appreciate the author's responses to my comments. The new version is improved but, in my opinion, still needs more work.

These revisions are changes to text, interpretations and obtaining more data from existing data or databases. I do still think one experimental control is necessary to substantiate the authors claim about membrane potential.

https://doi.org/10.7554/eLife.111002.1.sa3

Reviewer #2 (Public review):

This manuscript reports the identification of putative orthologues of mitochondrial contact site and cristae organizing system (MICOS) proteins in Plasmodium falciparum - an organism that unusually shows an acristate mitochondrion during the asexual part of its life cycle and then develops cristae as it enters the sexual stage of its life cycle and beyond into the mosquito. The authors identify PfMIC60 and PfMIC19 as putative members and study these in detail. The authors add HA tags to both proteins and look for timing of expression during the parasite life cycle and attempt (unsuccessfully) to localise them within the parasite - lack of signal concluded to be reflect very low expression levels. They also genetically delete both genes singly and in parallel and phenotype the effect on parasite development. They show that both proteins are expressed in gametocytes and not asexuals, suggesting they are present at the same time as cristae development. They also show that the proteins are dispensable for the entire parasite life cycle investigated (asexuals through to sporozoites), however there is some reduction in mosquito transmission. Using mitotracker labelling, the authors observe differences in mitochondrial organisation in gametocytes compared to the transgenic lines. Further investigation at higher resolution using EM techniques, shows data supporting their hypothesis that PfMIC60 and PfMIC19 are important for organising the parasite mitochondrion.

The manuscript is interesting and is an intriguing use of a well-studied organism of medical importance to answer fundamental biological questions. Given the essentiality of mitochondrial respiration for parasite survival in the mosquito, it is surprising that the single and double knock-out transgenics do not give a severe phenotype. However, the authors have been rigorous in characterizing the impact of genetic deletion of both genes throughout the parasite life cycle. Subtle differences in mitochondrial organisation were observed, consistent with their hypothesis that PfMIC60 and PfMIC19 play roles in mitochondrial organisation. Therefore, these data presented give new insights into an organelle that dramatically changes during parasite development and adds to our knowledge of mitochondrial biology in a highly unusual organism.

Comments on revised version:

I previously reviewed this manuscript for Review Commons. This version is greatly improved and the authors should be commended for addressing all comments raised.

https://doi.org/10.7554/eLife.111002.1.sa2

Reviewer #3 (Public review):

Summary:

MICOS is a conserved mitochondrial protein complex responsible for organising the mitochondrial inner membrane and the maintenance of cristae junctions. This study sheds

first light on the role of two MICOS subunits (Mic60 and the newly annotated Mic19) in the malaria parasite Plasmodium falciparum, which forms cristae de novo during sexual development, as demonstrated by EM of thin section and electron tomography. By generating knockout lines (including a double knockout), the authors demonstrate that knockout of both MICOS subunits leads to defects in cristae morphology and a partial loss of cristae junctions. With a formidable set of parasitological assays, the authors show that despite the metabolically important role of mitochondria for gametocytes, the knockout lines can progress through the life stages and form sporozoites, albeit with diminished infection efficiency.

Major comments (from the previous round of review):

(1) The authors should improve to present their findings in the right context, in particular by:

(i) giving a clearer description in the introduction of what is already known about the role of MICOS. This starts in the introduction, where one main finding is missing: loss of MICOS leads to loss of cristae junctions and the detachment of cristae membranes, which are nevertheless formed, but become membrane vesicles. This needs to be clearly stated in the introduction to allow the reader to understand the consistency of the authors' findings in P. falciparum with previous reports in the literature.

(ii) at the end to the introduction, the motivating hypothesis is formulated ad hoc "conclusive evidence about its involvement in the initial formation of cristae is still lacking" (line 83). If there is evidence in the literature that MICOS is strictly required for cristae formation in any organism, then this should be explained, because the bona fide role of MICOS is maintenance of cristae junctions (the hypothesis is still plausible and its testing important).

(2) Line 96-97: "Interestingly, PfMIC60 is much larger than the human MICOS counterpart, with a large, poorly predicted N-terminal extension." This statement is lacking a reference and presumably refers to annotated ORFs. The authors should clarify if the true N-terminus is definitely known - a 120kDa size is shown for the P. falciparum, but this is not compared to the expected length or the size in S. cerevisiae.

(3) lines 244-245: "Furthermore, our data indicates the effect size increases with simultaneous ablation of both proteins?". The authors should explain which data they are referring to, as some of the data in Figs 3 and 4 look similar and all significance tests relate to the wild type, not between the different mutants, so it is not clear if any overserved differences are significant. The authors repeat this claim in the discussion in lines 368-369 without referring to a specific significance test. This needs to be clarified.

(4) lines 304-306: "Though well established as the cristae organizing system, the role of MICOS in initial formation of cristae remains hidden in model organisms that constitutively display cristae.". This sentence is misleading since even in organisms that display numerous cristae throughout their life cycle, new cristae are being formed as the cells proliferate. Thus, failure to produce cristae in MICOS knockout lines would have been observable but has apparently not been reported in the literature. Thus, the concerted process in P. falciparum makes it a great model organism, but not fundamentally different to what has been studied before in other organisms.

(5) lines 373-378. "where ablation of just MIC60 is sufficient to deplete functionality of the entire MICOS (11, 15),". The authors' claim appears to be contrary to what is actually stated in ref 15, which they cite:

"MICOS subunits have non-redundant functions as the absence of both MICOS subcomplexes results in more severe morphological and respiratory growth defects than deletion of single MICOS subunits or subcomplexes."

This seems in line with what the authors show, rather than "different".

(6) lines 380-385: "... thus suggesting that membrane invaginations still arise but are not properly arranged in these knockout lines. This suggests that MICOS either isn't fully depleted,...". These conclusions are incompatible with findings from ref. 15, which the authors cite. In that study, the authors generated a ΔMICOS line which still forms membrane invaginations, showing that MICOS is not required at all for this process in yeast. Hence the authors' implication that MICOS needs to be fully depleted before membrane invaginations cease to occur is not supported by the literature.

(7) The authors should consider if the first part of their title could be seen as misleading: It suggests that MICOS is "the architect" in cristae formation, but this is not consistent with the literature nor their own findings.

Significance:

The main strength of the study is that it provides the first characterisation of the MICOS complex in P. falciparum, a human parasite in which the mitochondrion has been shown to be a drug target. Mic60 and the newly annotated Mic19 are confirmed to be essential for proper cristae formation and morphology, as well as overall mitochondrial morphology. Furthermore, the mutant lines are characterised for their ability to complete the parasite life cycle and defects in infection effectivity are observed. This work is an important first step for deciphering the role of MICOS in the malaria parasite and the composition and function of this complex in this organism.

The limitation of the study stems from what is already known about MICOS and its subunits in other organisms. MICOS subunit knockouts have been characterised in great detail in yeast and humans with similar findings regarding loss of cristae and cristae defects. The findings of this study do not provide dramatic new insight on MICOS function or go substantially beyond the vast existing literature in terms of the extent of the study, which focuses on parasitological assays and morphological analysis.

Exploring the role of MICOS in an early-divergent organism and human parasite is however important given the divergence found in mitochondrial biology and P. falciparum is a uniquely suited model system. One aspect that would increase the impact of the paper would be if the authors could mechanistically link the observed morphological defects to the decreased infection efficiency, e.g. by probing effects on mitochondrial function. This will likely be challenging as the morphological defects are diverse and the fitness defects appear moderate/mild.

The advance presented in this study is to pioneer the study of MICOS in P. falciparum, thus widening our understanding of the role of this complex to different model organism. This study will likely be mainly of interest for specialised audiences such as basic research parasitologists and mitochondrial biologists. My own field of expertise is mitochondrial biology and structural biology.

Comments on revised version:

The authors have addressed my all of my previous comments in the updated manuscript version.

https://doi.org/10.7554/eLife.111002.1.sa1

Author response:

> ***Reviewer #1 (Evidence, reproducibility and clarity):***

> *Summary:*
>
> *This manuscript reports the identification of putative orthologues of mitochondrial contact site and cristae organizing system (MICOS) proteins in Plasmodium falciparum - an organism that unusually shows an acristate mitochondrion during the asexual part of its life cycle and then this develops cristae as it enters the sexual stage of its life cycle and beyond into the mosquito. The authors identify PfMIC60 and PfMIC19 as putative members and study these in detail. The authors at HA tags to both proteins and look for timing of expression during the parasite life cycle and attempt (unsuccessfully) to localise them within the parasite. They also genetically deleted both gene singly and in parallel and phenotyped the effect on parasite development. They show that both proteins are expressed in gametocytes and not asexuals, suggesting they are present at the same time as cristae development. They also show that the proteins are dispensible for the entire parasite life cycle investigated (asexuals through to sporozoites), however there is some reduction in mosquito transmission. Using EM techniques they show that the morphology of gametocyte mitochondria is abnormal in the knockout lines, although there is great variation.*
>
> *Major comments:*
>
> *The manuscript is interesting and is an intriguing use of a well studied organism of medical importance to answer fundamental biological questions. My main comments are that there should be greater detail in areas around methodology and statistical tests used. Also, the mosquito transmission assays (which are notoriously difficult to perform) show substantial variation between replicates and the statistical tests and data presentation are not clear enough to conclude the reduction in transmission that is claimed. Perhaps this could be improved with clearer text?*

We would like to thank the reviewer for taking the time to review our manuscript. We are happy to hear the reviewer thinks the manuscript is interesting and thank the reviewer for their constructive feedback.

To clarify the statistical analyses used, we included a new supplementary dataset with all statistical analyses and p-values indicated per graph. Furthermore, figure legends now include the information on the exact statistical test used in each case.

Regarding mosquito experiments, while we indeed reported a reduction in transmission and oocysts numbers, we are aware that this effect might be due to the high variability in mosquito feeding assays. To highlight this point, we deleted the sentence “with the transmission reduction of [numbers]….” and we included the sentence “The high variability encountered in the standard membrane feeding assays, though, partially obstructs a clear conclusion on the biological relevance of the observed reduction in oocyst numbers“

> *More specific comments to address:*
>
> *Line 101/Fig1E (and figure legend) - What is this heatmap showing. It would be helpful to have a sentence or two linking it to a specific methodology. I could not find details in the M+M section and "specialized, high molecular mass gels" does not adequately explain what experiments were performed. The reference to Supplementary Information 1 also did not provide information.*

We added the information “high molecular mass gels with lower acrylamide percentage” to clarify methodology in the text. Furthermore, we extended the figure legend to include all relevant information. Further experimental details can be found in the study cited in this context, where the dataset originates from (Evers et al., 2021).

> *Line 115 and Supplementary Figure 2C + D - The main text says that the transgenic parasites contained a mitochondrially localized mScarlet for visualization and localization, but in the supplementary figure 2 it shows mitotracker labelling rather than mScarlet. This is very confusing. The figure legend also mentions both mScarlet and MitoTracker. I assume that mScarlet was used to view in regular IFAs (Fig S2C) and the MitoTracker was used for the expansion microscopy (Fig S2D)?*
>
> *Please clarify.*

We thank the reviewer for pointing this out – this was indeed incorrectly annotated. We used the endogenous mito-mScarlet signal in IFA and mitoTracker in U-ExM. The figure annotation has now been corrected.

> *Figure 2C - what is the statistical test being used (the methods say "Mean oocysts per midgut and statistical significance were calculated using a generalized linear mixed effect model with a random experiment effect under a negative binomial distribution." but what test is this?)?*

The statistic test is now included in the material and method section with the sentence “The fitted model was used to obtain estimated means and contrasts and were evaluated using Wald Statistics”. The test is now also mentioned in the figure legend.

> *Also the choice of a log10 scale for oocyst intensity is an unusual choice - how are the mosquitoes with 0 oocysts being represented on this graph? It looks like they are being plotted at 10^-1 (which would be 0.1 oocysts in a mosquito which would be impossible).*

As the data spans three orders of magnitude with low values being biologically meaningful, we decided that a log scale would best facilitate readability of the graph. As the 0 values are also important to show, we went with a standard approach to handle 0s in log transformed data and substituted the 0s with a small value (0.001). We apologize for not mentioning this transformation in the manuscript. To make this transformation transparent, we added a break at the lower end of the log-scaled y-axis and relabelled the lowest tick as ‘0’. This ensures that mosquitoes with zero oocysts are shown along the x-axis without being assigned an artificial value on the log scale. We would furthermore like to highlight that for statistics we used the true value 0 and not 0.001.

> *Figure 2D - it is great that the data from all feeding replicates has been shared, however it is difficult to conclude any meaningful impact in transmission with the knock-out lines when there is so much variation and so few mosquitoes dissected for some datapoints (10 mosquitoes are very small sample sizes). For example, Exp1 shows a clear decrease in mic19- transmission, but then Exp2 does not really show as great effect. Similarly, why does the double knock out have better transmission than the single knockouts? Sure there would be a greater effect?*

We agree with the reviewer and with the new sentence added, as per major point, we hope we clarified the concept. Note that original Figure 2D has been moved to the supplementary information, as per minor comment of another reviewer.

> *Figure 3 legend - Please add which statistical test was used and the number of replicates.*

Done

> *Figure 4 legend - Please add which statistical test was used and the number of replicates.*

Done. Regarding replicates, note that while we measured over 100 cristae from over 30 mitochondria, these all stem from the same parasite culture.

> *Figure 5C - the 3D reconstructions are very nice, but what does the red and yellow coloring show?*

Indeed, the information was missing. We added it to the figure legend.

> *Line 352 - "Still, it is striking that, despite the pronounced morphological phenotype, and the possibly high mitochondrial stress levels, the parasites appeared mostly unaffected in life cycle propagation, raising questions about the functional relevance of mitochondria at these stages."*
>
> *How do the authors reconcile this statement with the proven fact that mitochondria-targeted antimalarials (such as atovaquone) are very potent inhibitors of parasite mosquito transmission?*

Our original sentence was reductive. What we wanted to state was related to the functional relevance of crista architecture and overall mitochondrial morphology rather than the general functional relevance of the mitochondria. We changed the sentence accordingly.

Furthermore, even though we do not discuss this in the article, we are aware of mitochondria targeting drugs that are known to block mosquito transmission. We want to point out that it is difficult to discern the disruption of ETC and therefore an impact on energy conversion with the impact on the essential pathway of pyrimidine synthesis, highly relevant in microgamete formation. Still, a recent paper from Sparkes et al. 2024 showed the essentiality of mitochondrial ATP synthesis during gametogenesis so it is very likely that the mitochondrial energy conversion is highly relevant for transmission to the mosquito.

> ***Reviewer #1 (Significance):***
>
> *This manuscript is a novel approach to studying mitochondrial biology and does open a lot of unanswered questions for further research directions. Currently there are limitations in the use of statistical tests and detail of methodology, but these could be easily be addressed with a bit more analysis/better explanation in the text.*
>
> *This manuscript could be of interest to readers with a general interest in mitochondrial cell biology and those within the specific field of Plasmodium research.*
>
> *My expertise is in Plasmodium cell biology.*

We thank the reviewer for the praise.

> ***Reviewer #2 (Evidence, reproducibility and clarity):***
>
> *Major comments:*
>
> *(1) In my opinion, the authors tend to sensationalize or overinterpret their results. The title of the manuscript is very misleading. While MICOS is certainly important for crista formation, it is not the only factor, as ATP synthase dimer rows make a highly significant contribution to crista morphology. Thus, one can argue with equal validity that ATP synthase should be considered the 'architect', as it's the conformation of the dimers and rows modulate positive curvature. Secondly, while cristae are still formed upon mic60/mic19 gene knockout (KO), they are severely deformed, and likely dysfunctional (see below). Thus, I do not agree with the title that MICOS is dispensable for crista formation, because the authors results show that it clearly is essential. So, the title should be changed.*

We thank the reviewer for taking the time to review our manuscript.

Based on the reviewers' interpretation we conclude the title does not come across as intended. We have changed the title to: "The role of MICOS in organizing mitochondrial cristae in malaria parasites"

> *The Discussion section starting from line 373 also suffers from overinterpretation as well as being repetitive and hard to understand. The authors infer that MICOS stability is compromised less in the single KOs (sKO) in compared to the mic60/mic19 double KO (dKO). MICOS stability was never directly addressed here and the composition of the MICOS complex is unaddressed, so it does not make sense to speculate by such tenuous connections. The data suggest to me that mic60 and mic19 are equally important for crista formation and crista junction (CJ) stabilization, and the dKO has a more severe phenotype than either KO, further demonstrating neither is epistatic.*

We do agree with the reviewer's notion that we did not address complex stability, and our wording did not make this sufficiently clear. We shortened and rephrased the paragraph in question.

> *The following paragraphs (line 387 to 422) continues with such unnecessary overinterpretation to the point that it is confusing and contradictory. Line 387 mentions an 'almost complete loss of CJs' and then line 411 mentions an increase in CJ diameter, both upon Mic60 ablation. I do not think this discussion brings any added value to the manuscript and should be shortened. Yes, maybe there are other putative MICOS subunits that may linger in the KOS that are further destabilized in the dKO, or maybe Mic60 remains in the mic19 KO (and vice versa) to somehow salvage more CJs, which is not possible in the dKO. It is impossible to say with confidence how ATP synthase behaves in the KOs with the current data.*

We shortened this paragraph.

> *(2) While the authors went through impressive lengths to detect any effect on lifecycle progression, none was found except for a reduction in oocyte count. However, the authors did not address any direct effect on mitochondria, such as OXPHOS complex assembly, respiration, membrane potential. This seems like a missed opportunity, given the team's previous and very nice work mapping these complexes by complexome profiling. However, I think there are some experiments the authors can still do to address any mitochondrial defects using what they have and not resorting to complexome profiling (although this would be definitive if it is feasible):*
>
> *i) Quantification of MitoTracker Red staining in WT and KOs. The authors used this dye to visualize mitochondria to assay their gross morphology, but unfortunately not to assay membrane potential in the mutants. The authors can compare relative intensities of the different mitochondria types they categorized in Fig. 3A in 20-30 cells to determine if membrane potential is affected when the cristae are deformed in the mutants. One would predict they are affected.*

Interesting suggestion. As our staining and imaging conditions are suitable for such analysis (as demonstrated by Sarazin et al., 2025, *https://www.biorxiv.org/content/10.1101/2025.11.27.690934v1*), we performed the measurements on the same dataset which we collected for Figure 3. We did, however, not detect any difference in mitotracker intensity between the different lines. The result of this analysis is included in the new version of Supplementary figure S6.

> *ii) Sporozoites are shown in Fig S5. The authors can use the same set up to track their motion, with the hypothesis that they will be slower in the mutants compared to WT due to less ATP. This assumes that sporozoite mitochondria are active as in gametocytes.*

While theoretically plausible and informative, we currently do not know the relevance of mitochondrial energy conversion for general sporozoite biology or specifically features of sporozoite movement. Given the required resources and time to set this experiment up and the uncertainty whether it is a relevant proxy for mitochondrial functioning, we argue it is out of scope for this manuscript.

> *iii) Shotgun proteomics to compare protein levels in mutants compared to WT, with the hypothesis that OXPHOS complex subunits will be destabilized in the mutants with deformed cristae. This could be indirect evidence that OXPHOS assembly is affected, resulting in destabilized subunits that fail to incorporate into their respective complexes.*

While this experiment could potentially further our understanding of the interaction between MICOS and levels of OXPHOS complex subunits we argue that the indirect nature of the evidence does not justify the required investments.

> *To expedite resubmission, the authors can restrict the cell lines to WT and the dKO, as the latter has a stronger phenotype that the individual KOs and conclusions from this cell line are valid for overall conclusions about Plasmodium MICOS.*
>
> *I will also conclude that complexome/shotgun proteomics may be a useful tool also for identifying other putative MICOS subunits by determining if proteins sharing the same complexome profile as PfMic60 and Mic19 are affected. This would address the overinterpretation problem of point 1.*
>
> *(3) I am aware of the authors previous work in which they were not able to detect cristae in ABS, and thus have concluded that these are truly acristate. This can very well be true, or there can be immature cristae forms that evaded detection at the resolution they used in their volumetric EM acquisitions. The mitochondria and gametocyte cristae are pretty small anyway, so it not unreasonable to assume that putative rudimentary cristae in ABS may be even smaller still. Minute levels of sampled complex III and IV plus complex V dimers in ABS that were detected previously by the authors by complexome profiling would argue for the presence of miniscule and/or very few cristae.*
>
> *I think that authors should hedge their claim that ABS is acristate by briefly stating that there still is a possibility that miniscule cristae may have been overlooked previously.*

We acknowledge that we cannot demonstrate the absolute absence of any membrane irregularities along the inner mitochondrial membrane. At the same time, if such structures were present, they would be extremely small and unlikely to contain the full set of proteins characteristic of mature cristae. For this reason, we consider it appropriate to classify ABS mitochondria as acristate. To reflect the reviewer's point while maintaining clarity for readers, we have slightly adjusted our wording in the manuscript, changing 'fully acristate' to 'acristate'.

> *This brings me to the claim that Mic19 and Mic60 proteins are not expressed in ABS. This is based on the lack of signal from the epitope tag; a weak signal is detected in gametocytes. Thus, one can counter that Mic19 and Mic60 are also expressed, but below the expression limits of the assay, as the protein exhibits low expression levels when mitochondrial activity is upregulated.*

We agree with the reviewer that the absence of a detectable epitope-tag signal does not definitively exclude low-level expression, and we have therefore replaced the term 'absent' with 'undetectable' throughout the manuscript. In context with previous findings of low-level transcripts of the proteins in a study by Lopez-Berragan et al. and Otto et al., we also added the sentence "The apparent absence could indicate that transcripts are not translated in ABS or that the proteins' expression was below detection limits of western blot analysis." to the

discussion. At the same time, we would like to clarify that transcript levels for both genes fall within the <25th percentile, suggesting that these low values likely represent background signal rather than biologically meaningful expression. This interpretation is further supported by proteomic datasets in PlasmoDB, which report PfMIC19 and PfMIC60 expression in gametocyte and mosquito stages, but not in asexual blood stages."

> *To address this point, the authors should determine of mature mic60 and mic19 mRNAs are detected in ABS in comparison to the dKO, which will lack either transcript. RT-qPCR using polyT primers can be employed to detect these transcripts. If the level of these mRNAs are equivalent to dKO in WT ABS, the authors can make a pretty strong case for the absence of cristae in ABS.*

We appreciate the reviewer's suggestion. As noted in the Discussion, existing transcriptomic datasets already show detectable MIC19 and MIC60 mRNAs in ABS. For this reason, we expect RT-qPCR to reveal low (but not absent) levels of both transcripts, unlike the true loss expected to be observed in the dKO. Because such residual signals have been reported previously and their biological relevance remains uncertain, we do not believe transcript levels alone can serve as a definitive indicator of cristae absence in ABS.

> *They should highlight the twin CX9C motifs that are a hallmark of Mic19 and other proteins that undergo oxidative folding via the MIA pathway. Interestingly, the Mia40 oxidoreductase that is central to MIA in yeast and animals, is absent in apicomplexans (DOI: 10.1080/19420889.2015.1094593).*

Searching for the CX9C motifs is a valuable suggestion. In response to the reviewerś suggestion we analysed the conservation of the motif in PfMIC19 and included this in a new figure panel (Figure 1 F).

> *Did the authors try to align Plasmodium Mic19 orthologs with conventional Mic19s? This may reveal some conserved residues within and outside of the CHCH domain.*

In response to this comment we made Figure 1 F, where we show conserved residues within the CHCH domains of a broad range of MIC19 annotated sequences across the opisthokonts, and show that the Cx9C motifs are conserved also in PfMIC19. Outside the CHCH domain, we did not find any meaningful conservation, as PfMIC19 heavily diverges from opisthokont MIC19.

> *(5) Statistical significance. Sometimes my eyes see population differences that are considered insignificant by the statistical methods employed by the authors, eg Fig. 4E, mutants compared to WT, especially the dKO. Have the authors considered using other methods such as student t-test for pairwise comparisons?*

The graphs in figures 3, 4 and 5 got a makeover, such that they now are in linear scale and violin plots (also following a suggestion from further down in the reviewer's comments). We believe that this improves interpretability. ANOVA was kept as statistical testing to assure the correction for multiple comparisons that cannot be performed with standard t-test. A full overview of statistics and exact pvalues can also be found in the newly added supplementary information 2.

> *Minor comments:*
>
> *Line 33. Anaerobes (eg Giardia) have mitochondria that do produce ATP, unlike aerobic mitochondria*

We acknowledge that producing ATP via OXPHOS is not a characteristic of all mitochondria-like organelles (e.g. mitosomes), which is why these are typically classified separately from canonical mitochondria. When not considering mitochondria-like organelles, energy

conversion is the function that the mitochondrion is most well-known for and the one associated with cristae.

> *Line 56: Unclear what authors mean by "canonical model of mitochondria"*

To clarify we changed this to “yeast or human” model of mitochondria.

> *Lines 75-76: This applies to Mic10 only*

We removed the “high degree of conservation in other cristate eukaryotes” statement.

> *Line 80: Cite DOI: 10.1016/j.cub.2020.02.053*

Done

> *Fig 2D: I find this table difficult to read. If authors keep table format, at least get rid of 'mean' column' as this data is better depicted in 2C. I suggest depicted this data either like in 3B depicting portion of infected vs unaffected flies in all experiments, then move modified Table to supplement. Important to point out experiment 5 appears to be an outlier with reduced infectivity across all cell lines, including WT.*

To clarify: the mean reported in the table indicates the mean per replicate while the mean reported in figure 2C is the overall mean for a given genotype that corrects for variability within experiments. We agree that moving the table to the supplementary data is a good idea. We decided to not include a graph for infected and non-infected mosquitoes as this information would be partially misleading, highlighting a phenotype we argue to be influenced by the strong variability.

> *Fig. 3C-G: I feel like these data repeatedly lead to same conclusions. These are all different ways of showing what is depicted in Fig 2B: mitochondria gross morphology is affected upon ablation of MICOS. I suggest that these graphs be moved to supplement and replaced by the beautiful images.*

Thank you for the nice comment on our images. We have now moved part of the graphs to supplementary figure 6 and only kept the Relative Frequency, Sphericity and total mitochondria volume per cell in the main figure.

> *Line 180: Be more specific with which tubulin isoform is used as a male marker and state why this marker was used in supplemental Fig S6.*

We have now specified the exact tubulin isoform used as the male gametocyte marker, both in the main text and in Supplementary Fig. S6. This is a commercial antibody previously known to work as an effective male marker, which is why we selected it for this experiment. This is now clearly stated in the manuscript.

> *Line 196 and Fig 3C: the word 'intensities' in this context is very ambiguous. Please choose a different term (puncta, elements, parts?). This is related to major point 2i above.*

To clarify the biological effect that we can conclude form the measurement, we added an explanation about it in the respective section of the results, and we decided to replace the raw results of the plug-in readout with the deduced relative dispersion.

> *Line 222: Report male/female crista measurements*

We added Supplementary information 2, which contains exact statistical test and outcomes on all presented quantifications as well as a per-sex statistical analysis of the data from figure 4. Correspondingly, we extended supplementary information 2 by a per-sex colour code for the thin section TEM data.

> *Fig. 4B-E: depict data as violin plots or scatter plots like Fig. 2C to get a better grasp of how the crista coverage is distributed. It seems like the data spread is wider in the double KO. This would also solve the problem with the standard deviation extending beyond 0%.*

We changed this accordingly.

> *Lines 331-333: Please clarify that this applies for some, but not all MICOS subunits. Please also see major point 1 above. Also, the authors should point out that despite their structural divergence, trypanosomal cryptic mitofilins Mic34 and Mic40 are essential for parasite growth, in contrast to their findings with PfMic60 (DOI: https://doi.org/10.1101/2025.01.31.635831).*

This has been changed accordingly.

> *Line 320: incorrect citation. Related to point 1above.*

Correct citation is now included in the text.

> *Lines 333-335. This is related to the above. Again, some subunits appear to affect cell growth under lab conditions, and some do not. This and the previous sentence should be rewritten to reflect this.*

This has been changed accordingly.

> *Line 343-345: The sentence and citation 45 are strange. Regarding the former, it is about CHCHD10, whose status as a bona fide MICOS subunit is very tenuous, so I would omit this. About the phenomenon observed, I think it makes more sense to write that Mic60 ablation results in partially fragmented mitochondria in yeast (Rabl et al., 2009 J Cell Biol. 185: 1047-63). A fragmented mitochondria is often a physiological response to stress. I would just rewrite as not to imply that mitochondrial fission (or fusion) is impaired in these KOs, or at least this could be one of several possibilities.*

The sentence has been substituted following the indication of the reviewer. Though we still include the data of the human cells as this has also been shown in Stephens et al. 2020.

> *Line 373: 'This indicates' is too strong. I would say 'may suggest' as you have no proof that any of the KOs disrupts MICOS. This hypothesis can be tested by other means, but not by penetrance of a phenotype.*

Done

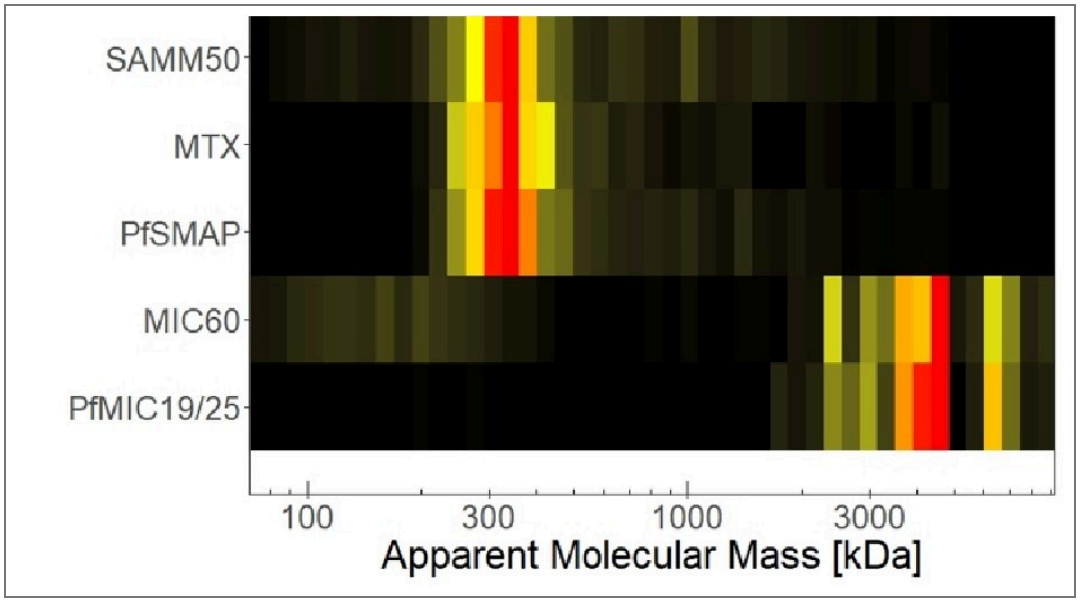
> *Line 376-377; 'deplete functionality' does not make sense, especially in the context of talking about MICOS subunit stability. In my opinion, this paragraph overinterprets the KO effects on MICOS stability. None of the experiments address this phenomenon, and thus the authors should not try to interpret their results in this context. See major point 1.*

We removed the sentence. Also, the entire paragraph has been shortened, restructured and wording was changed to address major point 1.

> *Other suggestions for added value*
>
> *(1) Does Plasmodium Sam50 co-fractionate with Mic60 and Mic19 in BN PAGE (Fig. 1E)*

While we did identify SAMM50 in our BN PAGE, the protein does not co-migrate with the MICOS components but instead comigrates with other components of a putative sorting and assembly machinery (SAM) complex. As SAMM50, the SAM complex and the overarching

putative mitochondrial membrane space bridging (MIB) complex are not mentioned in the manuscript, we decided to not include the information in Author response image 1.



Author response image 1.

Reviewer #2 (Significance):

The manuscript by Tassan-Lugrezin is predicated on the idea that Plasmodium represents the only system in which de novo crista formation can be studied. They leverage this system to ask the question whether MICOS is essential for this process. They conclude based on their data that the answer is no, which the authors consider unprecedented. But even if their claim is true that ABS is acristate, this supposed advantage does not really bring any meaningful insight into how MICOS works in Plasmodium.

First the positives of this manuscript. As has been the case with this research team, the manuscript is very sophisticated in the experimental approaches that are made. The highlights are the beautiful and often conclusive microscopy performed by the authors. Only the localization of Mic60 and Mic19 was inconclusive due to their very low expression unfortunately.

The examination of the MICOS mutants during in vitro life cycle of Plasmodium falciparum is extremely impressive and yields convincing results. Mitochondrial deformation is tolerated by life cycle stage differentiation, with a modest but significant reduction of oocyte production, being observed.

However, despite the herculean efforts of the authors, the manuscript as it currently stands represents only a minor advance in our understanding of the evolution of MICOS, which from the title and focus of the manuscript, is the main goal of the authors.

In its current form, the manuscript reports some potentially important findings:

(1) Mic60 is verified to play a role in crista formation, as is predicted by its orthology to other characterized Mic60 orthologs.

(2) The discovery of a novel Mic19 analog (since the authors maintain there is no significant sequence homology), which exhibits a similar (or the same?) complexome

profile with Mic60. This protein was upregulated in gametocytes like Mic60 and phenocopies Mic60 KO.

(3) Both of these MICOS subunits are essential (not dispensable) for proper crista formation

(4) Surprisingly, neither MICOS subunit is essential for in vitro growth or differentiation from ABS to sexual stages, and from the latter to sporozoites. This says more about the biology of plasmodium itself than anything about the essentiality of Mic60, i.e. plasmodium life cycle progression tolerates defects to mitochondrial morphology. But yes, I agree with the authors that Mic60's apparent insignificance for cell growth in examined conditions does differ with its essentiality in other eukaryotes. But fitness costs were not assayed (e.g. by competition between mutants and WT in infection of mosquitoes)

(5) Decreased fitness of the mutants is implied by a reduction of oocyte formation.

While interesting in their own way, collectively they do not represent a major advance in our understanding of MICOS evolution. Furthermore, the findings bifurcate into categories informing MICOS or Plasmodium biology. Both aspects are somewhat underdeveloped in their current form.

This is unfortunate because there seem to be many missed opportunities in the manuscript that could, with additional experiments, lead to a manuscript with much wider impact. For me, what is remarkable about Plasmodium MICOS that sets it apart from other iterations is the apparent absence of the Mic10 subunit. Purification of plasmodium MICOS via the epitope tagged Mic60 and Mic19 could have verified that MICOS is assembled without this core subunit. Perhaps Mic60 and Mic19 are the vestiges of the complex, and thus operate alone in shaping cristae. Such a reduction may also suggest the declining importance of mitochondria in plasmodium.

Another missed opportunity was to assay the impact of MICOS-depletion of OXPHOS in plasmodium.

This is a salient issue as maybe crista morphology is decoupled from OXPHOS capacity in Plasmodium, which links to the apparent tolerance of mitochondrial morphology in cell growth and differentiation. I suggested in section A experiments to address this deficit.

Finally, the authors could assay fitness costs of MICOS-ablation and associated phenotypes by assaying whether mosquito infectivity is reduced in the mutants when they are directly competing with WT plasmodium. Like the authors, I am also surprised that MICOS mutants can pass population bottlenecks represented by differentiation events. Perhaps the apparent robustness of differentiation may contribute plasmodium's remarkable ability to adapt.

I realize that the authors put a lot of efforts into their study and again, I am very impressed by the sophistication of the methods employed. Nevertheless, I think there is still better ways to increase the impact of the study aside from overinterpreting the conclusions from the data. But this would require more experiments along the lines I suggest in Section A and here.

We thank the reviewer for their extensive analysis of the significance of our findings, including the compliments on our microscopy images and the sophisticated experimental approaches. We hope we have convincingly argued why we could or could not include some of the additional analyses suggested by the reviewer in section 1 above.

With regard to the significance statement, we want to point out that our finding that PfMICOS is not needed for initial formation of cristae (as opposed to organization thereof), is a confirmation of something that has been assumed by the field, without being the actual focus of studies. We argue that the distinction between formation and organization of cristae is important and deserves some attention within the manuscript. The result of MICOS not being involved in the initial formation of cristae, we argue to be relevant in Plasmodium biology and beyond. As for the insights into how MICOS works in Plasmodium we have confirmed that the previously annotated PfMIC60 is indeed involved in the organization of cristae. Furthermore, we have identified and characterized PfMIC19. These findings, we argue, are indeed meaningful insights into PfMICOS.

> ***Reviewer #3 (Evidence, reproducibility and clarity):***
>
> *Summary:*
>
> *MICOS is a conserved mitochondrial protein complex responsible for organising the mitochondrial inner membrane and the maintenance of cristae junctions. This study sheds first light on the role of two MICOS subunits (Mic60 and the newly annotated Mic19) in the malaria parasite Plasmodium falciparum, which forms cristae de novo during sexual development, as demonstrated by EM of thin section and electron tomography. By generating knockout lines (including a double knockout), the authors demonstrate that knockout of both MICOS subunits leads to defects in cristae morphology and a partial loss of cristae junctions. With a formidable set of parasitological assays, the authors show that despite the metabolically important role of mitochondria for gametocytes, the knockout lines can progress through the life stages and form sporozoites, albeit with diminished infection efficiency.*

We thank the reviewer for their time and compliment.

> *Major comments:*
>
> *(1) The authors should improve to present their findings in the right context, in particular by:*
>
> *i) giving a clearer description in the introduction of what is already known about the role of MICOS. This starts in the introduction, where one main finding is missing: loss of MICOS leads to loss of cristae junctions and the detachment of cristae membranes, which are nevertheless formed, but become membrane vesicles. This needs to be clearly stated in the introduction to allow the reader to understand the consistency of the authors' findings in P. falciparum with previous reports in the literature.*

We extended the introduction to include this information.

> *iii) at the end to the introduction, the motivating hypothesis is formulated ad hoc "conclusive evidence about its involvement in the initial formation of cristae is still lacking" (line 83). If there is evidence in the literature that MICOS is strictly required for cristae formation in any organism, then this should be explained, because the bona fide role of MICOS is maintenance of cristae junctions (the hypothesis is still plausible and its testing important).*

To clarify we rephrased the sentence to: “Although MICOS has been described as an organizer of crista junctions, its role during the initial formation of nascent cristae has not been investigated.”

> *(2) Line 96-97: "Interestingly, PfMIC60 is much larger than the human MICOS counterpart, with a large, poorly predicted N-terminal extension." This statement is lacking a reference and presumably refers to annotated ORFs. The authors should clarify*

> *if the true N-terminus is definitely known - a 120kDa size is shown for the P. falciparum but this is not compared to the expected length or the size in S. cerevisiae.*

To solve the reference issue, we added the uniprot IDs we compared to see that the annotated ORF is bigger in Plasmodium. We also changed the comparison to yeast instead of human, because we realized it is confusing to compare to yeast all throughout the figure, but then talk about human in this specific sentence.

Regarding whether the true N-terminus is known. Short answer: No, not exactly.

However, we do know that the Pf version is about double the size of the yeast protein.

As the reviewer correctly states, we show the size of 120kDa for the tagged protein in Figure 1G. Considering that we tagged the protein C-terminally, and observed a 120kDa product on western blot, it is safe to conclude that the true N-terminus does not deviate massively from the annotated ORF, and hence, that there is a considerable extension of the protein beyond a 60kDa protein. We do not directly compare to yeast MIC60 on our western blots, however, that comparison can be drawn from literature: Tarasenko et al., 2017 showed that purified MIC60 running at ~60kDa on SDS-PAGE actively bends membranes, suggesting that in its active form, the monomer of yeast MIC60 is indeed 60kDa in size.

To clarify, we now emphasize that we ran the Alphafold prediction on the annotated open reading frame (annotated and sequenced by Bohme et al. and Chapell et al. now cited in the manuscript), and revised the wording to make clear what we are comparing in which sentence.

> *(3) lines 244-245: "Furthermore, our data indicates the effect size increases with simultaneous ablation of both proteins?". The authors should explain which data they are referring to, as some of the data in Fig 3 and 4 look similar and all significance tests relate to the wild type, not between the different mutants, so it is not clear if any overserved differences are significant. The authors repeat this claim in the discussion in lines 368-369 without referring to a specific significance test. This needs to be clarified.*

As a reply to this and other comments from the reviewers we added the multiple testing within all samples. In addition, to clarify statistics used we included a supplementary dataset with all p-values and statistical tests used.

> *(4) lines 304-306: "Though well established as the cristae organizing system, the role of MICOS in initial formation of cristae remains hidden in model organisms that constitutively display cristae.". This sentence is misleading since even in organisms that display numerous cristae throughout their life cycle, new cristae are being formed as the cells proliferate. Thus, failure to produce cristae in MICOS knockout lines would have been observable but has apparently not been reported in the literature. Thus, the concerted process in P. falciparum makes it a great model organism, but not fundamentally different to what has been studied before in other organisms.*

We deleted this statement.

> *(5) lines 373-378. "where ablation of just MIC60 is sufficient to deplete functionality of the entire MICOS (11, 15),". The authors' claim appears to be contrary to what is actually stated in ref 15, which they cite:*
>
> *"MICOS subunits have non-redundant functions as the absence of both MICOS subcomplexes results in more severe morphological and respiratory growth defects than deletion of single MICOS subunits or subcomplexes."*
>
> *This seems in line with what the authors show, rather than "different".*

This sentence has been removed.

> *(6) lines 380-385: "... thus suggesting that membrane invaginations still arise, but are not properly arranged in these knockout lines. This suggests that MICOS either isn't fully depleted,...". These conclusions are incompatible with findings from ref. 15, which the authors cite. In that study, the authors generated a ΔMICOS line which still forms membrane invaginations, showing that MICOS is not required at all for this process in yeast. Hence the authors' implication that MICOS needs to be fully depleted before membrane invaginations cease to occur is not supported by the literature.*

This sentence has been deleted in the revised version of the manuscript.

> *Minor comments:*
>
> *(1) The authors should consider if the first part of their title could be seen as misleading: It suggests that MICOS is "the architect" in cristae formation, but this is not consistent with the literature nor their own findings.*

Title is changed accordingly

> *- Line 43, of the three seminal papers describing the discovery of MICOS in 2011, the authors only cite two (refs 6 and 7), but miss the third paper, Hoppins et al, PMID: 21987634, which should probably be corrected.*

Done, the paper is now cited

> *- Page 2, line 58: for a more complete picture the authors should also cite the work of others here which shows that although at very low levels, e.g. complex III (a drug target) and ATP synthase do assemble (Nina et al, 2011, JBC).*

Done

> *- Page 3, line 80: "Irrespective of the shape of an organism's cristae, the crista junctions have been described as tubular channels that connect the cristae membrane to the inner boundary membrane (22, 24)." This omits the slit-shaped cristae junctions found in yeast (Davies et al, 2011, PNAS), which the authors should include.*

The paper and concept have been added to the manuscript, though the sentence has been moved up in the introduction, when crista junctions are first introduced.

> *- Line 97: "poorly predicted N-terminal extension", as there is no experimental structure, we don't know if the prediction is poor. Presumably the authors mean either poorly ordered or the absence of secondary structure elements, or the poor confidence score for that region in the prediction? This should be clarified or corrected.*

We were referring to the poor confidence score. To address this comment as well as major point 2, we rewrote the respective paragraph. It now clearly states that confidence of the prediction is low, and we mention the tool that was used to identify conserved domains (Topology-based Evolutionary Domains).

> *- Line 98: "an antiparallel array of ten β-sheets". They are actually two parallel beta-sheets stacked together. The authors could find out the name of this fold, but the confidence of the prediction is marked a low/very low. So, its existence is unknown, not just its "function".*

We adapted the domain description to “a stack of two parallel beta-sheets" and replaced the statement on unknown function by the statement “Because this domain is predicted solely from computational analysis, both its actual existence in the native protein and its biological function remain unknown.”

> *- Fig 1B: The authors show two alphafold predictions of S. cerevisiae and P. falciparum Mic60 structures. There is however an experimental Mic60/19 (fragment) structure from the former organism (PMID: 36044574), which should be included if possible.*

We appreciate the reviewer’s suggestion and note that the available structural data indeed provides valuable insight into how MIC60 and MIC19 interact. However, these structures represent fusion constructs of limited protein fragments and therefore capture only a small portion of each protein, specifically the interaction interface. Because our aim in Fig. 1B is to compare the overall domain architecture of the full-length proteins, we believe that including fragment-based structures would be less informative in this context.

> *- Line: 318-321: "The same trend was observed for PfMIC19 and PfMIC60. Although transcriptomic data suggested that low-level transcripts of PfMIC19 and PfMIC60 are present in ABS (38), we did not detect either of the proteins in ABS by western blot analysis. While this statement is true, the authors should comment on the sensitivity of the respective methods - how well was the antibody working in their hands and how do they interpret the absence of a WB band compared to transcriptomics data?*

The HA antibody used in our experiments is a standard commercial reagent that performs reliably in both WB and IFA, although it shows a low background signal in gametocytes. We agree that the sensitivity of the method and the interpretation of weak or absent bands should be addressed explicitly. Transcript levels for both PfMIC19 and PfMIC60 in asexual blood stages fall within the <25 percentile, suggesting that these signals likely represent background. Nevertheless, we acknowledge that low-level protein expression below the detection limit of western blot analysis cannot be excluded. To reflect these considerations, we added the sentence: ‘The apparent absence could indicate that transcripts are not translated in ABS or that the proteins’ expression was below detection limits of western blot analysis.

> *- Lines 322-323: would the authors not typically have expected an IFA signal given the strength of the band in Western blot? If possible, the authors should comment if the negative fluorescence outcome can indeed be explained with the low abundance or if technical challenges are an equally good explanation.*

Considering the nature of the investigated proteins (embedded in the IMM and spread throughout the mitochondria) difficulties in achieving a clear signal in IFA or U-ExM are not very surprizing. While epitopes may remain buried in IFA, U-ExM usually increases accessibility for the antibodies. However, U-ExM comes at the cost of being prone to dotty background signals, therefore potentially hiding low abundance, naturally dotty signals such as the signal of MICOS proteins that localize to distinct foci (at the CJ) along the mitochondrion. Current literature suggests that, in both human and yeast, STED is the preferred method for accurate spatial resolution of MICOS proteins (*https://www.ncbi.nlm.nih.gov/pubmed/32567732,https://www.ncbi.nlm.nih.gov/pubmed/32067344*). Unfortunately, we do not have experience with, nor access to, this particular technique/method.

> *- Lines 357-365: the authors describe limitations of the applied methods adequately. Perhaps it would be helpful to make a similar statement about the analysis of 3D objects like mitochondria and cristae from 2D sections. E.g. the apparent cristae length depends on whether cristae are straight (e.g. coiled structures do not display long cross sections despite their true length in 3D).*

The limitations of other methods are described in the respective results section.

We added a clarifying sentence in the results section of Figure 4:

“Note that such measurements do not indicate the true total length or width of cristae, as the data is two-dimensional. The recorded values are to be considered indicative of possible trends, rather than absolute dimensions of cristae.“

This statement refers to the length/width measurements of cristae.

In the context of Figure 4D we mention the following (see preprint lines 229 – 230): “We expect this effect to translate into the third dimension and thus conclude that the mean crista volume increases with the loss of either *PfMIC19*, *PfMIC60*, or both.”

For Figure 5, we included a clarifying statement in the results section of the preprint (lines 269 – 273): “Note that these mitochondrial volumes are not full mitochondria, but large segments thereof. As a result of the incompleteness of the mitochondria within the section, and the tomography specific artefact of the missing wedge, we were unable to confirm whether cristae were in fact fully detached from the boundary membrane, or just too long to fit within the observable z-range.”

> *- Line 404: perhaps undetected or similar would be a better description than "hidden"?*

The sentence does not exist in the revised manuscript.

> ***Reviewer #3 (Significance):***
>
> *The main strength of the study is that it provides the first characterisation of the MICOS complex in P. falciparum, a human parasite in which the mitochondrion has been shown to be a drug target. Mic60 and the newly annotated Mic19 are confirmed to be essential for proper cristae formation and morphology, as well as overall mitochondrial morphology. Furthermore, the mutant lines are characterised for their ability to complete the parasite life cycle and defects in infection effectivity are observed. This work is an important first step for deciphering the role of MICOS in the malaria parasite and the composition and function of this complex in this organism. The limitation of the study stems from what is already known about MICOS and its subunits in great detail in yeast and humans with similar findings regarding loss of cristae and cristae defects. The findings of this study do not provide dramatic new insight on MICOS function or go substantially beyond the vast existing literature in terms of the extent of the study, which focuses on parasitological assays and morphological analysis. Exploring the role of MICOS in an early-divergent organism and human parasite is however important given the divergence found in mitochondrial biology and P. falciparum is a uniquely suited model system. One aspect that would increase the impact of the paper would be if the authors could mechanistically link the observed morphological defects to the decreased infection efficiency, e.g. by probing effects on mitochondrial function. This will likely be challenging as the morphological defects are diverse and the fitness defects appear moderate/mild.*

As suggested by Reviewer 2, we examined mitochondrial membrane potential in gametocytes using MitoTracker staining and did not observe any obvious differences associated with the morphological defects. At present, additional assays to probe mitochondrial function in P.

falciparum gametocytes are not sufficiently established, and developing and validating such methods would require substantial work before they could be applied to our mutant lines. For these reasons, a more detailed mechanistic link between the observed morphological changes and the reduced infection efficiency is currently beyond reach.

> *The advance presented in this study is to pioneer the study of MICOS in P. falciparum, thus widening our understanding of the role of this complex to different model organism. This study will likely be mainly of interest for specialised audiences such as basic research parasitologists and mitochondrial biologists. My own field of expertise is mitochondrial biology and structural biology.*

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