Mitochondrial ATP synthase plays a key role in inducing membrane curvature to establish cristae. In Apicomplexa causing diseases such as malaria and toxoplasmosis, an unusual cristae morphology has been observed, but its structural basis is unknown. Here, we report that the apicomplexan ATP synthase assembles into cyclic hexamers, essential to shape their distinct cristae. Cryo-EM was used to determine the structure of the hexamer, which is held together by interactions between parasite-specific subunits in the lumenal region. Overall, we identified 17 apicomplexan-specific subunits, and a minimal and nuclear-encoded subunit-α.

The hexamer consists of three dimers with an extensive dimer interface that includes bound cardiolipins and the inhibitor IF1. Cryo-ET and subtomogram averaging revealed that hexamers arrange into ~20-megadalton pentagonal pyramids in the curved apical membrane regions. Knockout of the linker protein ATPTG11 resulted in the loss of pentagonal pyramids with concomitant aberrantly shaped cristae. Together, this demonstrates that the unique macromolecular arrangement is critical for the maintenance of cristae morphology in Apicomplexa.
F-type ATP synthases are energy-converting membrane protein complexes that synthesize adenosine triphosphate (ATP) from ADP and inorganic phosphate. These universal enzymes function by using the energy stored in an electrochemical potential across the bioenergetic membrane by rotary catalysis. The soluble $F_i$ complex and membrane-bound $F_o$ subcomplex together form the $F_iF_o$ ATP synthase monomer, which is found in bacteria and chloroplasts. In mitochondria, $F_iF_o$ ATP synthase resides in the crista membrane where it is known to form dimers, which can further assemble into rows critical for inducing the membrane curvature and maintaining membrane potential and morphology.

The main driving force for the synthesis of ATP in mitochondria is the membrane potential, which has been shown to be higher in the cristae lumen than in the adjacent intermembrane space. Cristae shaping has been shown to depend on the assembly of ATP synthase dimers into dimer rows, which is the basis for energy conversion in all mitochondria studied to date. However, the molecular interactions that convey the membrane-shaping properties of the oligomeric ATP synthase are poorly understood. Furthermore, structural data has shown that cristae morphology varies between eukaryotic lineages.

The infectious apicomplexan parasite Toxoplasma gondii is commonly used as a model organism for the malaria-causing agent Plasmodium spp. These parasites have a unique bulbous cristae morphology, which differs substantially from the lamellar cristae of their mammalian hosts. The underlying mechanism for the bulbous cristae is unknown. Loss of ATP synthase is accompanied by parasite death and defects in cristae abundance in the $T. gondii$ stage responsible for acute toxoplasmosis, and results in the death of the Plasmodium mosquito form responsible for malaria spread.

Here, we investigate the mechanism for the generation of the unique cristae in the Apicomplexa, using a combination of single-particle cryo-EM, cryo-ET and subtomogram averaging. We first report cristae-embedded ATP synthase hexamers arranged in pentagonal pyramids in the wild type, then identify a key subunit for the assembly, and finally characterise mutant cells with a generated knockout of this subunit.

Results

Structure of the hexameric ATP synthase and its herein identified elements. A large-scale preparation of $T. gondii$ tachyzoite mitochondria and subsequent mild solubilisation with digitonin resulted in the isolation of intact ATP synthase complexes, which we identified as native hexamers. We then performed solubilisation with $n$-dodecyl-$\beta$-D-maltoside ($\beta$-DDM) that resulted in dissociation of the hexamers into dimers. Both oligomeric forms were subjected to cryo-EM structure determination (Fig. 1, Supplementary Figs. 1 and 2). Masked refinements of the ATP synthase dimer resulted in maps of the membrane region, the OSCP/$F_i/c$-ring complex, the rotor and the peripheral stalk, ranging in resolution from 2.8 to 3.5 Å (Supplementary Figs. 1 and 3), thus allowing de novo modelling of the respective regions. Refinement into a 2.9-Å resolution consensus map allowed model construction of the entire ATP synthase dimer (Fig. 1a, b and Supplementary Table 1). The 1.85-MDa complex consists of 32 different subunits, of which only 15 are canonical with structural equivalents in other phyla. Homolog searches of 17 noncanonical subunits revealed them to be largely conserved in mitochondria. Apicomplexa including Plasmodium parasites, and in the related phyla of chromerids and perkinsozoa, suggesting that the herein described architecture is likely representative of myzozoans (Supplementary Fig. 4). Thus, following a species-specific nomenclature established in protozoan ATP synthases, we term the 17 apicomplexan-conserved $T. gondii$ subunits ATPTG1-17 (TG for $T. gondii$), with ATPTG1, ATPTG7, and ATPTG16 identified directly from the cryo-EM map (Supplementary Fig. 5a and Supplementary Table 2).

Parasite-specific subunits form a dimer interface that includes IF$_i$ and bound cardiolipins. The structure of the $T. gondii$ ATP synthase reveals that the unusual architecture of the dimer is generated by the peripheral stalks that are laterally offset, extending away from the central dimer axis (Fig. 1a, b). This architecture does not allow the formation of the conventional dimerization interface of type-I ATP synthases found in animals and yeast (Supplementary Fig. 5i, j), in which peripheral stalks extend along the dimer long axis. We therefore examined the dimerization interface, which is formed by the apicomplexan subunits and extensions of the canonical $F_o$ subunits. Those elements involve eleven proteins from each monomer that contribute more than 7000 Å$^2$ of buried surface area, making the interface substantially larger than in mammalian, yeast and algal ATP synthase structures (Supplementary Fig. 5c–j).

The dimerization interface in the membrane and luminal regions is governed by homotypic interactions between symmetry-related subunits, most of which extend deep into both monomers. Subunit-6 contains two transmembrane helices, each binds one of the symmetry-related subunit-a copies, which are therefore linked by four transmembrane helices (Fig. 2a, b). In addition, two cardiolipins are found on the matrix side, forming specific protein-lipid interactions bridging the two copies of subunit-b and subunit-f (Fig. 3a). This cardiolipin pair is sequestered in the $F_o$ subcomplex with no apparent path to the

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bulk membrane, suggesting a structural role. An additional 15 cardiolipins and 12 other phospholipids were found to mediate a network of interactions throughout the membrane region (Supplementary Fig. 6a). These native lipids are primarily bound in two vestibules within the Fo subcomplex (Fig. 3a) with the lipid head groups mediating charged interactions between numerous subunits (Supplementary Fig. 6b–e), which indicates a contribution to the stability of the complex.

The inhibitor protein of ATPase activity IF1 is bound to subunit-\(\beta\), contributing to the Fo dimer interface with its C-terminal helix extending from F1 to interact with subunit-\(\beta'\) of the neighbouring monomer (Fig. 3b). IF1 in our structure is bound exclusively to the \(\alpha/\beta\)-interface facing the dimer interface (Fig. 3b and Supplementary Fig. 7a–d, f), thereby locking it in the ADP-bound state (\(\beta_{\text{DP}}\)). The N-terminal IF1 region that contacts the central stalk in the bovine complex \(^2^5\) is absent in our structure (Supplementary Fig. 7b). Because central stalk rotation and conformational changes in the catalytic sites of F1 are interdependent, the sterically restrictive IF1 binding in \(T.\) gondii to only one of the three catalytic sites results in the trapping of the ATP synthase in a single rotational state in both the dimer and hexamer. In our cryo-EM maps, IF1 is contiguous with Fo-associated density extending to the C2-symmetry axis, thus linking the two F1 monomers (Fig. 2d, Supplementary Fig. 7f). We assign it to the unmodeled C-terminal region of IF1, which has previously been characterised as a homo-oligomerisation domain in mammals.\(^2^6\) This bridging of two F1 monomers is intra-dimeric, which is different from the mammalian ATP synthase tetramer, where bridging occurs between the two neighbouring dimers\(^2^4,2^7\) (Supplementary Fig. 7e, f).

**Evolutionary and functional aspects of a minimal and nuclear-encoded subunit-\(a\).** We assigned subunit-\(a\) by locating topologically conserved transmembrane helices of the canonical subunits \(b, d, f, i/j, k\), and 8 (Fig. 2a, b and Supplementary Fig. 8d). Based on the sequence identified directly from the cryo-EM map, we found that \(T.\) gondii subunit-\(a\) is encoded in the nucleus, and not in mitochondria as in most other organisms. Thus, in \(T.\) gondii, all ATP synthase subunits are nuclear-encoded (Supplementary Table 2). In addition, unlike in the canonical six-helix (H1-6\(a\)) fold, which is conserved in bacteria, chloroplasts and other mitochondria\(^4,2^8–3^0\), the subunit-\(a\) in \(T.\) gondii lacks H1-4\(a\), and only the horizontal H5\(a\) and H6\(a\) are found. They interact with the c-ring at the rotor-stator interface (Fig. 2a, b; Supplementary Fig. 8a, b). This is the smallest subunit-\(a\) structure reported to date.

The unmodelled sequence that would make up the canonical transmembrane H1-4\(a\) corresponds to a mitochondrial targeting sequence with a predicted cleavage site located N-terminally of...
contains only the conserved H5-6

The IF1-locked rotational state reveals salt bridge formation at mitochondrial energy conversion. 

three mitochondrial genes, while maintaining functional mitochondrial protein targeting following gene transfer to the nucleus. 

H5_ano, thereby causing the truncation (Supplementary Fig. 8c). Thus, compared to its mitochondria-encoded homologs, T. gondii subunit-a displays a reduced overall hydrophobicity, which we found to be conserved in Apicomplexa (Fig. 4a). A similar observation for different mitochondrial membrane proteins has been proposed to enable mitochondrial protein targeting.

The missing interactions of truncated H1-4, are compensated by lipids and apicomplexan subunits and extensions surrounding the canonical subunits, anchoring them to the enlarged Fo region and the wing region (Figs. 2b and 3a). Thus, the minimal T. gondii subunit-a exemplifies an evolutionary mechanism that combines subunit truncation and reduced hydrophobicity with structural compensation that allowed gene transfer. Our analyses illustrate how the substantial mitochondrial genome reduction occurred in apicomplexan parasites, retaining only three mitochondrial genes, while maintaining functional mitochondrial energy conversion.

The IF1-locked rotational state reveals salt bridge formation at the rotor-stator interface. In addition to minimal architecture and evolutionary insight, the subunit-a structure also reveals its interactions with the c-ring. The IF1-arrested structure, in which ATP synthases are locked in a single rotational state, allowed us to obtain a map of the rotor-stator interface at 3.5 Å (Supplementary Fig. 1d), resolving both the c-ring and H5-6 of subunit-a, where proton transfer occurs. Mechanistically, the essential arginine on H5_a (Arg166 in T. gondii) is thought to be responsible for deprotonation of the conserved glutamate on the c-ring (Glu150 in T. gondii). Translocating protons enter Fo via a luminal access channel, are transferred to the protonatable glutamate on the c-ring and released via a matrix channel. While our cryo-EM map does not display unambiguous density for Glu150, previous X-ray crystal structures have shown that this side chain can adopt an open unprotonated or closed proton-locked rotamer. Both formation and absence of a salt bridge between the arginine and glutamate have been observed in different structures, including a suggestion of a bridging water molecule.

Our structure indicates that in the open conformation Glu150 is within 2.3 Å distance from the juxtaposed Arg166, allowing the formation of a salt bridge (Fig. 4b). The rotor-stator interface surrounding the Arg166/Glu150 pair is more hydrophobic compared to other structures, with subunits a and c contributing a total of eight aromatic residue side chains (Fig. 4b, Supplementary Fig. 8e). Thus, the tight hydrophobic interface between the decameric c-ring and subunit-a in T. gondii is consistent with a direct, rather than water-mediated Arg/Glu interaction.

We traced two cavities in the Fo subcomplex corresponding to the proton half-channels on the luminal and matrix sides (Fig. 4c, d, Supplementary Fig. 8g). The luminal proton half-channel displays a hydrophilic entrance between subunit-a and ATPTG2 facing towards the c-ring (Fig. 4d, Supplementary Fig. 8f). Inside the membrane, the luminal channel is lined by membrane-inserted loops of ATPTG2 and ATPTG3 and the C-terminal transmembrane helix of subunit-b (Fig. 4d). The channel extends through the only acidic patch between H5_a and H6_a near a conserved glutamate (Glu201), which is thought to mediate proton transfer to the c-ring (Supplementary Fig. 8f). The matrix half-channel locates to a hydrophilic region between subunits a, d, ATPTG16, ATPTG17 and extends into the membrane region towards R159 of H5_ano, which is widely conserved (Fig. 4c). Remarkably, the C-terminus of ATPTG16 contributes...
Peripheral stalk subunit-\(b\) contains a structural motif found in the mammalian subunit F6. The peripheral stalk extends from the membrane-embedded part of \(F_0\), and attaches to the tip of \(F_1\), holding it stationary against the torque of the central stalk. In \(T. gondii\) the peripheral stalk is composed of subunit-\(b\), \(d\), ATPTG12 and OSCP (Fig. 1a, Supplementary Fig. 9b). The attachment to \(F_1\) is mediated through OSCP, which adopts a fold conserved in prokaryotic and eukaryotic homologs (Supplementary Fig. 9a). Subunit-\(b\) displays structural similarity with its bacterial, algal and mammalian counterparts, engaging in conserved interactions with the C-terminal domain of OSCP as observed in other structures. Compared to the yeast and mammalian ATP synthases, \(T. gondii\) displays an augmented peripheral stalk structure with extensions in subunit-\(b\), subunit-\(d\) and the additional ATPTG12 (Supplementary Fig. 9b). Unlike yeast and porcine\(^{24,39}\), neither subunit-\(f\) nor 8 (A6L in mammals) contribute to the peripheral stalk. Instead, the apicomplexan-conserved subunit ATPTG11 forms extensive interactions with subunit-\(b\) and \(d\) throughout the peripheral stalk structure (Supplementary Fig. 9b).

Interestingly, peripheral stalk subunit F6 (subunit \(h\) in yeast) is not found in \(T. gondii\) ATP synthase. Instead, the C-terminal extension of subunit-\(b\), adopts a fold that structurally resembles subunit F6/\(h\) and provides supporting interactions with the long subunit-\(b\) helix (Supplementary Fig. 9c). Both, the yeast subunit-\(h\) (on non-fermentable carbon sources) and the augmented \(T. gondii\) subunit-\(b\) are essential\(^{18,42}\), suggesting a critical role in peripheral stalk assembly.

**Formation of the ATP synthase hexamer involves two contact sites in the luminal region.** Next, we asked how ATP synthase dimers interact in the hexamer structure to form the ciliary trimer of dimers. The hexamer model shows that each of the three dimer–dimer interfaces contributes \(-1211\text{Å}^2\) to hexamer contacts. Those contacts holding the hexamer together are found in two separate sites in the luminal regions, which form a triangular subcomplex (Fig. 5a).

In the first site, three copies of the subunit ATPTG9 are arranged around the \(C_3\)-symmetry axis, directly beneath the central lipid bilayer (Supplementary Fig. 2e, f). ATPTG9 contains two CHCHDs with cysteine pairs positioned to form disulfide bonds. CHCHD-containing subunits were reported to play a role in the assembly of Complex IV\(^{43}\). In our structure, H2 of the first CHCHD in one ATPTG9 copy interacts with H5TG9 and the loop connecting H2 and H5TG9 of a neighbouring ATPTG9 (Fig. 5a). Both interacting structural elements are predominantly hydrophilic, consistent with their solvent-accessible location in the lumen.

In the second site, located at the periphery of the luminal region, ATPTG11 establishes a network of five interacting subunits. A helix hairpin of ATPTG11, containing a central cysteine pair, extends...
parallel to the membrane plane and mediates interactions between
the dimers. H2TG11 contacts with subunits ATPTG5, ATPTG8 and
ATPTG10, whereas H3TG11 interacts with ATPTG8 (Fig. 5b). Apart
from the inward-facing residues, the ATPTG11 helix hairpin and
interacting subunit segments are predominantly hydrophilic and
solvent-exposed in the dimer.

For ATPTG9, we found that the four CX9C motifs are
conserved in mitochondriate Apicomplexa (Supplementary
Fig. 10a, d). Likewise, we identified apicomplexan orthologs of
ATPTG5 and ATPTG11, including candidate genes in
Plasmodium with conserved cysteine pairs of the helix hairpins and key
residues, including the N-terminal helix of ATPTG11 (Supple-
mentary Fig. 10b, c, e, f). These data suggest that mitochondrial
ATP synthase hexamers are a common feature of Apicomplexa.

Hexamers specifically assemble into pentagonal pyramids in
curved membrane regions. To investigate if the mitochondrial
ATP synthase hexamers occur in situ, we performed cryo-ET of
isolated T. gondii mitochondrial membranes. Tomograms showed
that the inner-membrane vesicles frequently displayed bulbous
protrusions decorated with ATP synthase arrays (Fig. 6a, Sup-
plementary Movie 2). Using subtomogram averaging, we then
obtained a 20-Å resolution map of the ATP synthase dimer,
which agrees well with the atomic model (Supplementary
Fig. 11a, c). The analysis of the macromolecular arrangement of
ATP synthase dimers in the membrane suggested that they are
arranged into regular arrays with a hexamer as the repetitive unit,
confirming the occurrence of this cyclic oligomeric form in situ
(Fig. 6a).

Our data further show that these ATP synthase hexamers are
arranged in larger arrays with icosahedral symmetry. The most
frequently observed arrangement consists of ten ATP synthase
dimers arranged into five hexamer units, forming a pentagonal
pyramid (Fig. 6a and Supplementary Movie 2). In the pyramid,
the five inner dimers form a pentameric interface, with the C5
symmetry axis centred on the apex of the vesicular protrusion
(Fig. 5b). Each of the five inner dimers is shared by two
neighbouring hexamer units (Fig. 5b).

To understand how the pentagonal pyramid induces the
membrane curvature, we analysed cross-sections of the array
(Fig. 6b, d, e). This showed that while the lipid bilayer within
hexamers is near-planar (Supplementary Fig. 2e), neighbouring
hexamers planes are related by a 45° angle (Fig. 6d). This is
consistent with two times the 22° incline of each dimer with
respect to the C3 symmetry axis through the hexamer plane
(Figs. 1c, d and 5b), indicating that the single-particle hexamer
structure is consistent with the in situ pyramid assembly. Thus,
membrane curvature is induced locally around the five inner
dimers. Furthermore, hexamer planes are oriented by 40° with
respect to the pentamer plane formed in the centre of the
pyramid (Fig. 6a, b, e).

Fitting the dimer models into the pentagonal pyramid array
suggested that no additional contacts are formed at the pentamer
site. This indicates that the assembly of the pentagonal pyramids
is fully explained by the contacts between the luminal regions.
This involves the same interactions as in the hexamer (Figs. 5 and
6c). Due to the C2-symmetry of the dimer, each linker subunit is
present in two copies, allowing the propagation of interactions
(Fig. 6c), which results in the formation of a ~3.6-MDa array in
the cristae lumen (Fig. 6a).

Pentagonal pyramids are required for maintenance of native
cristae architecture. Toxoplasma tachyzoites are a model system
to study mitochondrial functions in Apicomplexa, as they can be
cultured using alternative energy sources to oxidative phosphorylation, thereby enabling the mutation of genes encoding proteins involved in mitochondrial energy conversion. To investigate the role of ATP synthase hexamers in maintaining native cristae architecture, we generated a knockout line of ATPTG11. Native gel electrophoresis confirmed that dimer assembly occurs in the absence of ATPTG11, and cryo-ET of mitochondrial membranes isolated from the ATPTG11-KO line revealed an altered organisation of the dimers in situ. Particularly, instead of forming pentagonal pyramids, ATP synthase dimers were found loosely arranged into disordered or row-like arrays along flat membrane regions. This demonstrates that ATP11-KO lumenal interfaces hold the pentagonal pyramids together. Visualization of the ATPTG11-KO mitochondrial membranes by cryo-ET revealed an elongated tubular shape, indicating that the formation of hexamers and pentagonal pyramids is critical for the maintenance of the bulbous cristae morphology in T. gondii. Thus, the ATPTG11-KO demonstrates the role of specific oligomer contacts in cristae architecture.

In addition, analysis of thin sections showed that ATPTG11-KO contains fewer cristae per mitochondrial area than the parental line, indicating an altered structure. Flow cytometry using the potential-sensitive fluorescent dye JC-1 indicated that the mitochondria of ATPTG11-KO remain energized by a mitochondrial membrane potential, which is sensitive to the ionophore valinomycin, like the parental line. Fluorescence microscopy further revealed that the single large mitochondrion of both lines forms the characteristic lasso-shape, indicating that overall mitochondrial ultrastructure is not affected. Finally, we performed a growth competition assay where ATPTG11-KO parasites were grown in a mixed population with the parental line. Quantitative PCR of isolated genomic DNA (gDNA) during continued culturing showed that the relative abundance of the ATPTG11-KO decreased significantly. These results indicate that the loss of ATP synthase oligomers and aberrant morphology are linked to impaired parasite fitness, when compared to the parental line.

In summary, we demonstrate that ATPTG11-KO selectively disrupts the formation of higher oligomers, while assembly of dimers appears unaffected. The resulting mild phenotype in cultured tachyzoites is in contrast to the strong growth defect that accompanies ATP synthase disassembly following loss of the indispensable core subunit-β. This suggests that T. gondii tachyzoites, which utilize both glycolysis and oxidative phosphorylation, can compensate for the aberrant macromolecular organisation of ATP synthase, but not for the complete loss of its catalytic function.

**Pentagonal pyramid arrays shape unique cristae of Toxoplasma mitochondria.** To confirm the occurrence of the pentagonal pyramids in *organello*, we performed cryo-ET of T. gondii mitochondria with a translucent matrix. The analysis showed that cristae display bulbous morphology and are attached to the inner boundary membrane by circular cristae junctions. The bulbous cristae protrusions are capped by apical arrays of ATP synthases arranged into the pentagonal pyramids. Thus, the apicomplexan cristae morphology is in stark contrast to the coiled tubular cristae found in the related phylum of ciliates, which are shaped by helical ATP synthase dimer rows and connected via one crista junction at either end.

In addition, recent cryo-EM structures of a ciliate ATP synthase dimer and tetramer showed that although both structures share a small dimer angle and a large luminal region, the two alveolate ATP synthases have diverged significantly and acquired lineage-specific subunits. Rather than forming hexamers, the ciliate-specific structural elements mediate dimer-dimer contacts that result in the formation of long helical ATP synthase.

**Fig. 6** T. gondii ATP synthase arranges into pentagonal pyramids with icosahedral symmetry to induce membrane curvature. a Cryo-ET of the mitochondrial membranes (blue) and subtomogram averaging of dimers reveals their macromolecular arrangement into pentagonal pyramids held together by proteins in the lumen. b Schematic representation shows five hexamers (coloured) arranged around a C5-axis. Red and blue arrows indicate cross-sections shown in the other panels. Inner and outer dimers are shown as red and black ellipses, respectively. c Schematic of interactions between luminal subunits involved in the assembly of the pentagonal pyramid. d Neighbouring hexamer planes (blue and red) are arranged at a ~45°-angle around the shared dimer (grey). e Cross section through the pentagonal ATP synthase pyramid showing two 40°-angles between hexamer (yellow, red) and pentamer (grey) planes.
Fig. 7 Pentagonal pyramids are required for maintenance of the native cristae morphology. a, b Parental strain (a) and ATPTG11-KO (b) cross sections of tomograms of mitochondrial membranes decorated with ATP synthase (yellow arrows). c, d Segmentation of mitochondrial membranes (blue) with repositioned subtomogram averages of the dimers (yellow). Whereas the parental strain forms pentagonal pyramids that cap the bulbous membrane protrusions, hexamer and pyramid formation is disrupted in ATPTG11-KO, and ATP synthase dimers arrange in row-like or disordered arrays along elongated or tubular membranes. Close-up views show the pentagonal pyramid in the parental strain and row-like arrangements in the mutant strain. e, f Relative abundances of parental (e) and ATPTG11-KO (f) of the mixed-culture growth competition assay as determined by qPCR of total gDNA, normalized to t0. Each passage represents 3–8 biological replicates; error bars are SD; p-values were determined by one-way ANOVA followed by Dunnett’s multiple comparisons test comparing each passage to P1.
dimer rows and cristae tubulation. Thus, the superphylum of alveolates contains at least two different types of ATP synthase dimers and cristae morphologies, differentiating the free-living and parasitic protist phyla.

Our data suggest that in the organellar context, the exclusive localisation of the ATP synthase in the curved membrane regions will result in its segregation from the respiratory chain complexes residing in the flat cristae regions\(^6\). Such preferential localisation of proton sinks has been suggested to generate a directional proton flow along a lateral proton gradient inside the cristae\(^4\). Together with the recent visualisation of cristae as high-potential compartments\(^12\), these results suggest that assembly of a membrane-shaping ATP synthase oligomer drives its localisation to regions of high membrane potential, thus favouring ATP synthesis.

In summary, this work demonstrates that ATP synthase can be arranged in previously unseen high oligomeric arrays, which differ from the spontaneously assembled dimer rows, that were thought to be universal in all mitochondria\(^5\). We describe an organisational principle based on specific interactions between ATP synthase hexamers that are assembled into a pentagonal pyramid architecture. This results in the induction of local membrane curvature, which gives rise to the unique bulbous cristae morphology in Apicomplexa.

**Methods**

**Cell culture and mitochondria isolation.** *T. gondii* RH tachyzoites were grown in Vero cells in DMEM supplemented with 10% (w/v) FBS, 2% (w/v) L-glutamine and 29.9 mM penicillin, 17.2 mM streptomycin at 37 °C with 5% (v/v) CO\(_2\). For each cell culture, parasites were harvested at >80% host-cell lysis and the media passed through 23G needles to fully lyse any remaining host cells. Parasites were pelleted by centrifugation at 1500 × g, 10 min, 4 °C, washed in PBS and then resuspended in buffer containing 210 mM mannitol, 70 mM sucrose, 50 mM HEPES-KOH pH 7.4, 1 mM EGTA, 5 mM EDTA, 10 mM KCl, 1 mM DTT to 5 × 10\(^9\) cells/ml. Parasites were lysed by successive rounds of nitrogen cavitation (2500 PSI, 15 min incubation on ice) until >95% lysis (confirmed by light microscopy). After each round, the lysate was centrifuged at 1500 × g, 15 min, 4 °C; the supernatant was collected and the pellet resuspended in the same volume for further lysis.

The final combined lysate was centrifuged as before and the supernatant was spun again at 16,000 × g, 30 min, 4 °C. The resulting crude mitochondrial pellet was further purified on a discontinuous sucrose gradient in 20 mM HEPES-KOH pH 7.4, 2 mM EDTA, 15/23/32/60% (w/v) sucrose by centrifugation (103,745 × g, 1 h, 4 °C) in an SW41 rotor (Beckman Coulter) and enriched mitochondria were collected from the 32–60% (w/v) interface.

**Real-time quantitative PCR.** To assess the expression level of ATPTG11 in the newly generated KO-line, ~5 × 10\(^6\) freshly lysed parasites were filtered through 3-μm filters and collected by centrifugation. RNA was extracted from parasite pellets using the RNeasy kit (Qiagen) following manufacturer’s instructions with the following modification: DNA was additionally on-column digested with 1 µl of ampicillin grade DNAse (ThermoFisher Scientific) for 15 min at RT during step 4 of the manufacturer’s protocol. Samples were reverse transcribed to cDNA using the High-Capacity RNA-to-cDNA kit (ThermoFisher Scientific) following manufacturer’s protocol. Twenty nanograms of cDNA were then used in each qPCR reaction, which was set up with Power SYBR Green Master Mix (ThermoFisher Scientific) with 300 nM of each primer. All qPCR reactions were performed using a 7800 Real-Time PCR System (Applied Biosystems) using default temperature settings and performing a dissociation step after each run. Relative gene expression was determined using the \(\Delta\Delta\)CT method\(^14\) using *T. gondii* catalase as an endogenous reference. The experiment was performed for ATPTG11-KO and the parental parasite line using primers against the unmodified ATPTG11 locus.
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**Growth competition assay.** A confluent HFF monolayer was inoculated with ~1×
ratio of ATPTG11-KO and parental parasites and incubated as described above. After complete host cell lysis, the collected parasites were mixed thoroughly, a new HFF was inoculated and the remaining parasites were filtered (3-µm pore size) and collected for gDNA extraction. gDNA was extracted using QiaGen DNeasy Blood & Tissue Kit. Power SYBR Green Master Mix, 300 ng of each primer and 10 ng of gDNA were used to perform quantitative PCR (7500 Real-Time PCR System using default settings with added dissociation step). The relative abundance of each parasite line was calculated relative to *T. gondii* calamine and normalized to the first collection point (t0) using the ΔΔct method66 using the primers against the native locus and DHFR. Data were analysed using GraphPad Prism 8.4.3.

**Immunofluorescence assay and microscopy.** Parasites were inoculated on confluent HFFs on glass coverslips. After 1 day-cells were fixed with 4% (w/v) paraformaldehyde. Cells were permeabilised and blocked with a 2% bovine serum albumin and 0.2% (w/v) triton X-100 in PBS before incubation with primary antibodies (rabbit anti-TgMys55; 1:1000, followed by secondary antibodies (Alexa Fluor Goat anti-Rabbit 594 Invitrogen #A-11012, 1:1000). Coverslips were mounted on slides with Fluoromount-G mounting medium containing DAPI (Southern Biotech, 0100-20). Images were acquired via a DeltaVision Core microscope (Applied Precision) using a x100 objective35. A total of seven representative images of ATPTG11-KO (containing individual vacuoles) and 10 images of parental parasites (containing 44 individual vacuoles) were obtained from two biologically independent repeats. Images were processed and deconvoluted using SoftWoRx (Glasgow, UK) and Fiji software36.

**Flow cytometry analysis of membrane potential using JC-1.** Parasites grown in HFF were allowed to lyse the host cells. Collected parasites were filtered through a 3-µm filter and incubated in their growth media with 10 µM valinomycin for 30 min at 37°C (as a depolarising control) or with an equal volume of DMSO, and then washed once with 5.6% Tetrachloro-4,3-tetra-ethylbenzimidazolocarbocyanine iodide, Thermo Fisher Scientific, stock 1.5 mM in DMSO) for 15 min at 37°C before analysis with the CytoFLEX (Beckman Coulter System using default settings with added dissociation step). The relative abundance of parental parasites (containing 44 individual vacuoles) were obtained from two biologically independent repeats. Images were processed and deconvoluted using FlowJo (FlowJo LLC) to visualise the population of parasites with red fluorescent signal.

**Blue-native polyacrylamide gel electrophoresis and immunoblotting.** Whole parasites (5 × 10^6) were mixed with 5 µM solubilisation buffer (750 mM amincaproic acid, 50 mM Bis-Tris–HCl pH 7.0, 0.5 mM EDTA, 1% (w/v) dodecyl maltoside) and incubated on ice for 10 min. The resulting lysate was centrifuged at 18,000 × g at 4 °C for 30 min. Sample buffer was added to the supernatant (NativePAGE® 5% (w/v) G-250 Sample Additive and NativePAGE® Sample Buffer (4X) (Invitrogen™), with a final concentration of Coomassie of 0.0625% (w/v), resulting in a final concentration of 0.25% (w/v) dodecyl maltoside. NativePAGE® Running Buffer (20X) and NativePAGE® Cathode Buffer Additive (20X (Invitrogen™) were mixed to 1:1 ratio, dark and light cathode buffers according to the manufacturer’s instructions. Samples were loaded on 3-12% (w/v) Bis-Tris Gel (Novex- Life technologies) and 5 µL NativeMark® (Invitrogen™) was used as a molecular weight marker. Gels were run for 1 h at 80 V, 10 mA at 4 °C with dark cathode buffer, and then for ~2 h at 200 V, 6 mA with light cathode buffer. Proteins were visualised from the gels on a PVDF membrane (0.45 µm, Hybond™). Wet transfer in Towbin buffer (0.025 M Tris 0.192 M glycine 10% (v/v) methanol) was performed for 60 min at 100 V. The membrane was stained with Coomassie solution (50% methanol, 7% (w/v) acetic acid, and 0.05% (w/v) Coomassie R250 (Serva)) to visualise the molecular weight marker, and destained with 50% methanol, 7% acetic acid. Blots were labelled with primary rabbit anti-ATP-β (1:2000, Agrisera) coupled to secondary horseradish peroxidase (HRP) anti-rabbit (Promega) conjugated antibodies (1:10,000) and visualised using the Pierce ECL Western Blotting Substrate (Thermo Scientific).

**Purification of *T. gondii* ATP synthase dimers and hexamers.** Enriched mitochondria were lysed in a total volume of 34 ml buffer containing 25 mM HEPES/ KOH pH 7.4, 2 mM EDTA, 250 mM sucrose and mixed to reconstitute the anode, dark and light cathode buffers according to the manufacturer’s instructions. A total of 7604 micrographs were acquired at a nominal magnification of 130 kx (1.05 Å/pixel) with a total exposure of 32 electrons/A^2 over 6.5 s, fractionated into 20 frames. Initial picking references were generated from the data for reference-based particle picking using Gautomatch. Subsequent image processing was described above on glow-discharged Quantifoil R2/2 Cu grids. Tilt series were acquired on a Titan Krios operated at 300 kV with a Quantum K2 camera (slit width 20 eV) using serialEM60 or the EPU software. Mitochondrial membranes were imaged at a nominal magnification of 64 kx (2.21 Å/pixel) and an exposure rate of 1.5 electrons/pixel/s with a 2× exposure fractionated into four frames with tilt series acquired using the exposure-symmetric scheme of tgamma60 tilt and a 3× tilt increment. Mitochondrial ghosts were imaged at a nominal magnification of 33 kx (4.27 Å/pixel) and an exposure rate of 11.5 electrons/pixel/s with a 3× exposure fractionated into 3 frames. Bidirectional tilt series were acquired from -60° to 60° starting at 24° with a 2° tilt increment and a defocus range of -5 to -8 Å. Frames were motion-corrected and exposure-weighted using MotionCor257 and CTF estimation was performed using Gctf58. Three Fo subunits were identified in the grid and used as references for particle picking. All resolution estimates were according to Fourier shell correlations (FSC) that were calculated from independently refined half-maps using the 0.143-criterion with correction for the effect of the applied masks (Supplementary Figs. 1 and 2).

**Electron cryo-tomography and subtomogram averaging.** Crude *T. gondii* mitochondria pellets from either the parental or ATPTG11-KO strain were resuspended in an equal volume of buffer containing 20 mM HEPES-KOH pH 7.4, 2 mM EDTA, 250 mM sucrose and mixed to reconstitute the anode, dark and light cathode buffers according to the manufacturer’s instructions. A total of 7604 micrographs were acquired at a nominal magnification of 130 kx (1.05 Å/pixel) with a total exposure of 32 electrons/A^2 over 6.5 s, fractionated into 20 frames. Initial picking references were generated from the data for reference-based particle picking using Gautomatch. Subsequent image processing was described above on glow-discharged Quantifoil R2/2 Cu grids. Tilt series were acquired on a Titan Krios operated at 300 kV with a Quantum K2 camera (slit width 20 eV) using serialEM60 or the EPU software. Mitochondrial membranes were imaged at a nominal magnification of 64 kx (2.21 Å/pixel) and an exposure rate of 1.5 electrons/pixel/s with a 2× exposure fractionated into four frames with tilt series acquired using the exposure-symmetric scheme of tgamma60 tilt and a 3× tilt increment. Mitochondrial ghosts were imaged at a nominal magnification of 33 kx (4.27 Å/pixel) and an exposure rate of 11.5 electrons/pixel/s with a 3× exposure fractionated into 3 frames. Bidirectional tilt series were acquired from -60° to 60° starting at 24° with a 2° tilt increment and a defocus range of -5 to -8 Å. Frames were motion-corrected and exposure-weighted using MotionCor257 and CTF estimation was performed using Gctf58. Three Fo subunits were identified in the grid and used as references for particle picking. All resolution estimates were according to Fourier shell correlations (FSC) that were calculated from independently refined half-maps using the 0.143-criterion with correction for the effect of the applied masks (Supplementary Figs. 1 and 2).

**Tomographic reconstruction was performed in IMOD62 using phaselipping3 and a binning factor 2. Tomograms were contrast enhanced using nonlinear anisotropic diffusion filtering68 to facilitate manual particle picking of ATP synthases. Subtomogram averaging was performed in PEErogen66. Initial references were generated from the data by averaging after rotating subvolumes into a common orientation with respect to the membrane. Following initial rounds of subtomogram averaging, false-positive particles were removed based on a cross-correlation coefficient cut-off and manually by visual inspection of their orientation (e.g. removal of upside-down particles). Particles were then split into odd and even halves using IMOD, then aligned in Gautomatch with a mask around the ATP synthase dimer. A 20-Å resolution map was obtained from 139 ATP synthase dimers from one tomogram of mitochondrial membranes (Supplementary Figs. 11 and 15b), whereas a 22-Å resolution map of the ATPTG11-KO dimer was obtained from 269 particles from two tomograms. A 34-Å resolution map was obtained from 410 ATP synthase dimers from one tomogram of a *T. gondii* calamine (Supplementary Fig. 15). Final maps were lowpass-filtered according to the 0.143-FSC criterion using RELION66.

**Atomic model building and refinement.** Manual building of atomic models was performed in Coot67. EF subunits were built de novo using reconstructions of Fe and peripheral stalk respectively (Supplementary Fig. 1). Built subunits were verified by BLAST searches against two libraries of putative *T. gondii* ATP synthase subunits (three EF, three Fe) identified by BLAST searches against built sequences against ToxoDB (toxdb.org) (Extended Data Table 1). OSCPF/c-ring models were built using a homology model69 of the yeast F1/c-ring (PDB ID 1J28) and visualised using the Coot67 software. Atomic coordinates and structure factors have been deposited in the Protein Data Bank (PDB ID 6OF5).
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Author contributions
A.M., L.S. and A.A. designed the project. A.M., P.F., A.L., J.O., L.S. and A.A. performed preparation of mitochondria from parasites. L.S. and J.O. generated the mutant line. A.M. performed protein purification and biochemical characterization, prepared cryo-EM grids, collected and processed EM data. A.M. and R.K.F. built, refined and validated the structures. A.M., R.K.F. and A.A. wrote the manuscript with help from L.S. All authors contributed to revising the manuscript.

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