

1 **Divergent features of the coenzyme Q:cytochrome *c* oxidoreductase complex in *Toxoplasma***
2 ***gondii* parasites**

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10 Running Title: Divergent Complex III in *Toxoplasma* parasites

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14 **Abstract**

15 The mitochondrion is critical for the survival of apicomplexan parasites. Several major anti-
16 parasitic drugs, such as atovaquone and endochin-like quinolones, act through inhibition of the
17 mitochondrial electron transport chain at the coenzyme Q:cytochrome *c* oxidoreductase complex
18 (Complex III). Despite being an important drug target, the protein composition of Complex III of
19 apicomplexan parasites has not been elucidated. Here, we undertake a mass spectrometry-based
20 proteomic analysis of Complex III in the apicomplexan *Toxoplasma gondii*. Along with canonical
21 subunits that are conserved across eukaryotic evolution, we identify several novel or highly
22 divergent Complex III components that are conserved within the apicomplexan lineage. We
23 demonstrate that one such subunit, which we term *TgQCR11*, is critical for parasite proliferation,
24 mitochondrial oxygen consumption and Complex III activity, and establish that loss of this protein
25 leads to defects in Complex III integrity. We conclude that the protein composition of Complex
26 III in apicomplexans differs from that of the mammalian hosts that these parasites infect.

27 **Author summary**

28 Apicomplexan parasites cause numerous diseases in humans and animals, including malaria
29 (*Plasmodium* species) and toxoplasmosis (*Toxoplasma gondii*). The coenzyme Q:cytochrome *c*
30 oxidoreductase protein complex (Complex III) performs a central role in the mitochondrial
31 electron transport chain of many eukaryotes. Despite being the target of several major anti-
32 apicomplexan drugs, the protein composition of Complex III in apicomplexans was previously
33 unknown. Our work identifies novel proteins in Complex III of apicomplexans, one of which is
34 critical for complex function and integrity. Our study highlights divergent features of Complex III
35 in apicomplexans, and provides a broader understanding of Complex III evolution in eukaryotes.
36 Our study also provides important insights into what sets this major drug target apart from the
37 equivalent complex in host species.

38

39 Introduction

40 Apicomplexans are a large phylum of intracellular, protozoan parasites that include the causative
41 agents of malaria (*Plasmodium* species) and toxoplasmosis (*Toxoplasma gondii*). These parasites
42 impose major economic and health burdens on human societies, and, in the absence of effective
43 vaccines [1, 2], there is a heavy reliance on drugs to treat disease. The parasite coenzyme
44 Q:cytochrome *c* oxidoreductase complex (Complex III of the mitochondrial electron transport
45 chain, ETC) represents one of the major drug targets in these parasites [3, 4]. Numerous inhibitors
46 of Complex III, including atovaquone and endochin-like quinolones, are in clinical use or in
47 preclinical development against apicomplexans [5-7].

48 The ETC consists of a series of protein complexes that are embedded in the inner mitochondrial
49 membrane. Electrons derived from the oxidation of mitochondrial substrates are donated via the
50 action of dehydrogenases to a mobile electron carrier in the inner membrane called coenzyme Q
51 (CoQ). CoQ exchanges electrons with the coenzyme Q:cytochrome *c* oxidoreductase complex
52 (Complex III). Here, electrons from CoQ are donated to the cytochrome *b* protein of Complex III,
53 from where they are either donated on to cytochrome *c*, a mobile carrier protein in the
54 mitochondrial intermembrane space, or donated back to CoQ in a process called the Q cycle [8].
55 The transfer of electrons to cytochrome *c* occurs via an iron-sulfur cluster and a heme prosthetic
56 group in the Rieske and cytochrome *c*₁ proteins of Complex III, respectively. Electrons from
57 cytochrome *c* are transported on to the cytochrome *c* reductase complex (Complex IV), which
58 facilitates electron transfer to the terminal electron acceptor, oxygen [9]. Electron transport through
59 Complexes III and IV is coupled to the translocation of protons from the mitochondrial matrix into
60 the intermembrane space, thereby generating a proton motive force across the inner membrane.

61 This proton gradient is used for several important mitochondrial processes, including protein and
62 solute import, and driving the activity of ATP synthase (Complex V) to generate ATP [10].

63 It is becoming increasingly apparent that the ETC of myzozoans – a eukaryotic lineage that
64 includes apicomplexan parasites and their closest free living relatives, two phyla of marine algae
65 called chromerids and dinoflagellates – differs considerably from that of other eukaryotes,
66 including the animal hosts that apicomplexans infect [11-13]. For instance, Complexes IV and V
67 from these organisms contain many subunits that lack homologs outside the myzozoan lineage
68 [14-16]. Uncovering such diversity in a canonical mitochondrial process is of interest from both
69 an evolutionary and a therapeutic standpoint. Evolutionarily, these studies provide insights into the
70 diversification of mitochondria since eukaryote lineages diverged from their common ancestor
71 >1.5 billion years ago [17]. Therapeutically, the discovery of novel proteins in a critical pathway
72 such as the ETC opens avenues for drug development.

73 Despite being a major drug target, many important features of Complex III in these parasites,
74 including its protein composition, have not yet been elucidated. Here, we undertake a proteomic
75 analysis of Complex III in *T. gondii*. Along with canonical subunits that are conserved in host
76 organisms, we identified two highly divergent and two novel, apicomplexan-specific Complex III
77 components. We demonstrate that one of the apicomplexan-specific subunits, which we term
78 *TgQCR11*, is critical for parasite proliferation and ETC function through maintaining Complex III
79 integrity. We conclude that Complex III of apicomplexans contains highly divergent and novel
80 protein subunits, and differs considerably from the equivalent complex in animal hosts.

81 Results

82 ***T. gondii* Complex III contains both canonical and novel protein subunits.** The putative
83 mitochondrial processing peptidase alpha subunit (*TgMPP α* ; www.toxodb.org gene ID
84 TGGT1_202680) was shown previously to localize to the mitochondrion of *T. gondii* [18]. In many
85 other eukaryotes, the MPP α protein functions in both the cleavage of mitochondrial-targeting pre-
86 sequences from mitochondrial matrix proteins as they are imported into the mitochondrion, and as
87 one of the so-called Core proteins of Complex III [19]. To facilitate further characterization of
88 *TgMPP α* , we introduced a TEV-HA tag into the 3' end of the open reading frame of the native
89 *TgMPP α* locus in *T. gondii* parasites (Fig. S1). To determine whether *TgMPP α* exists as part of a
90 protein complex, we extracted proteins from *TgMPP α* -TEV-HA parasites using either 1% (v/v)
91 Triton X-100, 1% (w/v) n-Dodecyl β -D-maltoside (DDM) or 1% (w/v) digitonin detergents, and
92 separated solubilized protein complexes by blue native (BN)-PAGE. Western blotting with anti-
93 HA antibodies revealed that the primary *TgMPP α* -TEV-HA complex is ~675 kDa in mass when
94 solubilized in all three detergents (Fig. 1A), though a fainter secondary complex at ~220 kDa was
95 also observed. By contrast, the molecular mass of *TgMPP α* -TEV-HA when separated by SDS-
96 PAGE was ~60 kDa (Fig. 1B). We conclude that *TgMPP α* is a component of a ~675 kDa protein
97 complex.

98 To identify the proteins that comprise the *TgMPP α* -containing complex, we immunoprecipitated
99 *TgMPP α* -TEV-HA and associated proteins, then subjected the sample to mass spectrometry-based
100 proteomic analysis. As a control for these experiments, we purified the unrelated cytochrome *c*
101 oxidase complex (Complex IV of the ETC). To purify Complex IV, we introduced a TEV-HA tag
102 into the 3' end of the cytochrome *c* oxidase subunit 2a (*TgCox2a*; TGGT1_226590) open reading
103 frame (Fig. S1), immunoprecipitated *TgCox2a*-TEV-HA and associated proteins, and performed a

104 separate mass spectrometry-based proteomic analysis. Using this approach, we identified five
105 proteins – including *TgMPP α* – that were enriched in the *TgMPP α* -TEV-HA immunoprecipitation
106 compared to the *TgCox2a*-TEV-HA immunoprecipitation across three independent experiments
107 (Fig. 1C, D; Table S1). All five proteins are annotated as canonical components of Complex III,
108 including the Core/mitochondrial processing peptidase protein *TgMPP β* (TGGT1_236210), the
109 iron-sulfur cluster protein *TgRieske* (TGGT1_320220), the so-called ‘14 kDa’ protein *TgQCR7*
110 (TGGT1_288750), and the cytochrome *c*₁ heme protein *TgCytC1* (TGGT1_246540). Two other
111 canonical Complex III proteins – the ‘hinge’ protein *TgQCR6* (TGGT1_320140) and the
112 cytochrome *b* protein (*TgCytB*; TGGT1_362110) – were highly enriched in the *TgMPP α*
113 immunoprecipitation but excluded from the initial analysis because they were absent from at least
114 one replicate of the *TgCox2a* control (Fig. 1D; Table S1). Note that the gene ID for *TgCytB*
115 (TGGT1_362110) encodes a truncated protein, and may represent one of several fragmented
116 *TgCytB* pseudogenes encoded by genetic material that occurs as ‘junk’ DNA in the nuclear
117 genome of *T. gondii*, rather than the actual mitochondrial genome-encoded *TgCytB* gene [20, 21].
118 Given that two canonical Complex III proteins were excluded from the initial analysis, we reasoned
119 that a more comprehensive assessment of the data might reveal additional subunits that were
120 likewise excluded. We shortlisted six additional proteins that were highly enriched in all three
121 *TgMPP α* replicates relative to *TgCox2a* (either absent from all *TgCox2a* samples or > 100-fold
122 more abundant in the *TgMPP α* purification than in the *TgCox2a* purification) (Fig. 1D; Table S1).
123 Of these six proteins, five were annotated as hypothetical proteins (TGGT1_201880,
124 TGGT1_227910, TGGT1_207170, TGGT1_214250, and TGGT1_242780) and one was annotated
125 as a GAF domain-containing protein (TGGT1_270800). We utilized Localization of Organelle
126 Proteins by Isotope Tagging (“LOPIT”) cellular localization data [13] coupled with our previously

127 published mitochondrial proteome [15] to assess whether these proteins are likely to be
128 mitochondrial. All the proteins were identified in both the mitochondrial proteome and the
129 mitochondrial membrane fraction of the “LOPIT” dataset, with the exception of TGGT1_242780,
130 which we excluded from further consideration (Fig. 1D, gray).

131 We employed a phylogenomic approach to further assess the candidate Complex III proteins. The
132 chromerid *Vitrella brassicaformis* contains the full suite of ETC complexes including Complex
133 III, while *Chromera velia* lacks Complex III but retains the other ETC complexes [12]. We
134 reasoned that if a protein has a homolog in *V. brassicaformis* but not in *C. velia*, this would support
135 the hypothesis that it is a Complex III subunit. In addition, the apicomplexan *Cryptosporidium*
136 *parvum* contains highly reduced mitochondria that lack Complex III [22]. We further hypothesized
137 that ‘true’ Complex III proteins were likely to be absent from *C. parvum*. To this end, we
138 performed tBLASTn searches of EuPathDB using the identified proteins as queries to identify
139 homologs in *Plasmodium falciparum*, *V. brassicaformis*, *C. velia* and *C. parvum* (Fig. 1D). Since
140 *TgMPP α* and *TgMPP β* function together as a mitochondrial peptidase protein complex, potentially
141 separate from their function in Complex III, it is unsurprising that these proteins are found in all
142 four species (Fig. 1D). In contrast, most other canonical Complex III proteins have homologs in
143 *P. falciparum* and *V. brassicaformis* but not in *C. velia* and *C. parvum*. An interesting exception
144 is the *TgRieske* protein, which has a homolog in *C. velia* (Fig. 1D). The *CvRieske* protein lacks
145 the long N-terminus typical of other Rieske proteins, but retains the iron-sulfur cluster-containing
146 core, implying this protein may have a secondary function in *C. velia*. A potential *TgCytC1*
147 homolog was also identified in *C. velia* (Fig. 1D); however, this matched only to the N-terminus
148 of *TgCytC1*, and lacked key residues including those that mediate heme binding. The *CvCytC1* is
149 likely not a functional CytC1 protein.

150 Of the additional proteins that we shortlisted, one has homologs in both chromerids
151 (TGGT1_270800) and was therefore excluded from further analysis, three have homologs in *V.*
152 *brassicaformis* but not *C. velia* (TGGT1_201880, TGGT1_227910, TGGT1_214250), and one
153 appeared to be restricted to *T. gondii* (TGGT1_207170) (Fig. 1D). Further analysis of
154 TGGT1_207170 using iterative JackHMMER homology searches identified a homolog of this
155 protein in *P. falciparum* and numerous other apicomplexans, but not in *C. parvum*, chromerids or
156 dinoflagellates (Fig. 1D). These data suggest that TGGT1_207170 may be an apicomplexan-
157 specific protein.

158 To investigate whether the four shortlisted proteins (Fig. 1D, green) have any similarity to known
159 Complex III proteins, we queried each protein against the Protein Data Bank (PDB) using
160 HHPRED, a profile hidden Markov model search tool designed to identify homologous proteins
161 with limited sequence similarity [23]. This analysis predicted that TGGT1_227910 has homology
162 to the yeast Complex III protein QCR8 and TGGT1_201880 has homology to yeast QCR9. We
163 therefore termed these two divergent Complex III subunits *TgQCR8* and *TgQCR9*, respectively
164 (Fig. 1D; Fig. S2; Fig S3). The remaining two proteins were not matched to proteins from PDB
165 with any confidence. In total, our proteomic analysis identified *T. gondii* homologs to nine out of
166 the ten proteins from the well-studied Complex III of budding yeast, with no homolog to the yeast
167 QCR10 protein apparent in *T. gondii*. In addition, we identified two proteins that were restricted
168 to the myzozoan or apicomplexan lineages. We termed these protein *TgQCR11* (TGGT1_214250)
169 and *TgQCR12* (TGGT1_207170), delineating them from the 10 “QCR” protein from yeast (Fig.
170 1D; Fig. S4; Fig S5). All the candidate Complex III proteins that were tested in a genome-wide
171 CRISPR screen were predicted to be important for growth of the tachyzoite stage of *T. gondii* [24]
172 (Fig. 1D).

173 To begin to characterize the candidate Complex III proteins experimentally, we introduced FLAG
174 epitope tags into the 3' end of the open reading frames of the *TgQCR8*, *TgQCR9*, *TgQCR11*, and
175 *TgQCR12* loci in an existing *TgMPP α -HA* background strain [18] (Fig. S6). We then undertook
176 western blot analysis and immunofluorescence assays to analyze the expression and cellular
177 localization of these proteins. All four proteins were found to be between 15-20 kDa in mass and
178 to localize to the mitochondrion (Fig. 2). Interestingly, the relative abundance of these four proteins
179 appeared to differ, with *TgQCR11-FLAG* and *TgQCR8-FLAG* the most abundant and *TgQCR12-*
180 *FLAG* the least (Fig. S7).

181 We next sought to determine whether *TgQCR11-FLAG*, *TgQCR8-FLAG*, *TgQCR9-FLAG* and
182 *TgQCR12-FLAG* exist in protein complexes. To do this, we extracted proteins from the four
183 parasite lines and from the *TgMPP α -TEV-HA* parasite line using 1% (w/v) DDM and separated
184 proteins by BN-PAGE. Western blotting using anti-FLAG antibodies revealed that all four proteins
185 exist in a protein complex of ~675 kDa, approximately the same mass as the *TgMPP α -TEV-HA*
186 complex when an adjacent lane run on the same gel was probed with anti-HA antibodies (Fig. 3A).

187 As a direct test for whether *TgQCR11-FLAG*, *TgQCR8-FLAG*, *TgQCR9-FLAG* and *TgQCR12-*
188 *FLAG* proteins are part of the same protein complex as *TgMPP α -HA*, we performed co-
189 immunoprecipitation experiments. Immunoprecipitation of *TgMPP α -HA* with anti-HA antibodies
190 co-purified each of the four FLAG-tagged proteins, but not the unrelated mitochondrial protein
191 *TgTom40* (Fig. 3B-E). Likewise, immunoprecipitation of *TgQCR11-FLAG* (Fig. 3B), *TgQCR8-*
192 *FLAG* (Fig. 3C), *TgQCR9-FLAG* (Fig. 3D) and *TgQCR12-FLAG* (Fig. 3E) with anti-FLAG
193 antibodies co-purified *TgMPP α -HA* but not *TgTom40*. In all instances, we identified a small
194 proportion of *TgMPP α -HA* in the unbound fraction of the anti-FLAG immunoprecipitations (Fig.
195 3B-D). This could represent the ~220 kDa complex observed for *TgMPP α -HA* in BN-PAGE that

196 is absent from the BN-PAGE analyses of the other proteins (Fig. 3A). Together, these data indicate
197 that *TgMPPα*-HA and the four QCR proteins exist in the same, ~675 kDa protein complex.

198 ***TgQCR11* is important for *T. gondii* proliferation and mitochondrial oxygen consumption.**

199 The existence of *TgQCR11*-FLAG, *TgQCR8*-FLAG, *TgQCR9*-FLAG and *TgQCR12*-FLAG in a
200 complex with the core protein *TgMPPα*-HA suggests that these proteins are components of
201 Complex III in *T. gondii*. To elucidate the importance and role of one of the apicomplexan-specific
202 Complex III proteins, we undertook a functional characterization of *TgQCR11*. We first added a
203 FLAG tag to the 3' end of the *TgQCR11* gene (Fig. S8A-B) and then replaced the native promoter
204 of *TgQCR11* with an anhydrotetracycline (ATc)-regulated promoter (Fig. S8C-D). We termed the
205 resultant ATc-regulated *TgQCR11* strain 'r*TgQCR11*-FLAG'. We also added an HA tag to the 3'
206 end of the *TgMPPα* gene in this strain (Fig. S8E-F), resulting in a strain we termed 'r*TgQCR11*-
207 FLAG/*TgMPPα*-HA'.

208 To examine the extent of *TgQCR11* knockdown upon the addition of ATc, we cultured r*TgQCR11*-
209 FLAG/*TgMPPα*-HA parasites in the absence of ATc or in the presence of ATc for 1-3 days, then
210 separated proteins by SDS-PAGE and performed western blotting. *TgQCR11*-FLAG expression
211 levels decreased substantially upon the addition of ATc, with only a small amount of protein
212 detectable after 3 days in ATc (Fig. 4A). To investigate the impact of *TgQCR11*-FLAG
213 knockdown on parasite proliferation, we grew wild type (WT) and r*TgQCR11*-FLAG/*TgMPPα*-
214 HA parasites in the absence or presence of ATc for 8 days and compared plaque sizes. Plaque sizes
215 in r*TgQCR11* but not WT parasites was severely impaired in the presence of ATc (Fig. 4B). To
216 determine whether this proliferation defect was specifically due to loss of *TgQCR11*, we
217 complemented the r*TgQCR11*-FLAG strain with an additional copy of *TgQCR11* expressed from
218 the constitutively expressed α -tubulin promoter. Complementation with constitutively expressed

219 *TgQCR11* restored plaque formation in *rTgQCR11* parasites cultured in the presence of ATc (Fig.
220 4B), indicating that the proliferation defect we observed upon *TgQCR11* knockdown was
221 specifically due to loss of *TgQCR11*.

222 Oxygen acts as the final electron acceptor in the ETC. If *TgQCR11* is an important component of
223 Complex III, we hypothesized that knockdown of *TgQCR11* knockdown would lead to defects in
224 oxygen consumption in the parasite. To test this, we utilized a previously established assay to
225 measure parasite oxygen consumption using a Seahorse XFe96 extracellular flux analyzer [15].
226 We grew WT and *rTgQCR11-FLAG/TgMPP α -HA* parasites in the absence of ATc or in the
227 presence of ATc for 1-3 days then measured their basal mitochondrial oxygen consumption rate
228 (mOCR). While the presence of ATc did not impair basal mOCR in WT parasites, the basal mOCR
229 of *rTgQCR11-FLAG* parasites was substantially depleted upon *TgQCR11* knockdown (Fig. 4C),
230 indicating that *TgQCR11* is important for mitochondrial oxygen consumption.

231 We wondered whether the severe deficiency in mOCR observed upon *TgQCR11* knockdown was
232 due to a specific defect in the ETC, or whether it was caused by a more general defect in
233 mitochondrial function or parasite viability. To test whether *TgQCR11* knockdown causes any
234 gross defects in mitochondrial morphology, we grew *rTgQCR11/TgMPP α -HA* parasites in the
235 absence or presence of ATc and performed immunofluorescence assays to visualize both the inner
236 (*TgMPP α -HA*) and outer (*TgTom40*) mitochondrial membranes. This revealed no observable
237 defects in mitochondrial morphology upon *TgQCR11* knockdown (Fig. S9A). Next, we tested
238 whether parasites remain viable following knockdown of *TgQCR11*. To do this, we pre-incubated
239 *rTgQCR11* parasites in the presence of ATc for 3 days (a time point when *TgQCR11* is knocked
240 down substantially and mOCR is minimal (Fig. 4)) and then set up plaque assays in the absence or
241 presence of ATc. After 8 days proliferation, we compared plaque size to parasites that had not been

242 pre-incubated in ATc. Plaque size and number were similar between the ATc-pre-incubated and
243 non-pre-incubated parasites when grown in the absence of ATc, while parasites grown in ATc
244 underwent minimal growth, regardless of whether they were pre-incubated with ATc (Fig. S9B).
245 These results indicate that *TgQCR11* knockdown is reversible and that *rTgQCR11* parasites grown
246 in ATc for 3 days have similar viability to parasites grown in the absence of ATc.

247 To observe what happens to other aspects of parasite metabolism upon *TgQCR11* knockdown, we
248 measured the extracellular acidification rate (ECAR) of WT and *rTgQCR11* parasites grown in the
249 absence or presence of ATc. We have previously used ECAR as a general indication of parasite
250 metabolic activity [15, 25]. ECAR levels of WT and *rTgQCR11* parasites grown in the absence of
251 ATc or WT parasites grown in the presence of ATc were not significantly different (Fig. 4D; Fig.
252 S9C). By contrast, growth of *rTgQCR11* parasites in the presence of ATc for 2 days resulted in a
253 significant *increase* in ECAR (Fig. 4D; Fig. S9C), indicating that parasites remained metabolically
254 active upon *TgQCR11* knockdown. The small but significant increase in ECAR upon *TgQCR11*
255 knockdown may indicate that the parasite compensates for loss of mOCR by upregulating other
256 aspects of cellular metabolism (e.g. glycolysis), though this needs to be studied further.

257 Together, these data indicate that the defects observed in mitochondrial oxygen consumption upon
258 *TgQCR11* knockdown were not due to general defects in mitochondrial morphology, parasite
259 viability or cellular metabolism. We therefore conclude that *TgQCR11* has an important and
260 specific role in the ETC of *T. gondii* parasites, consistent with its association with Complex III.

261 ***TgQCR11* is important for the function and integrity of Complex III.**

262 To establish whether *TgQCR11* is important for Complex III function in *T. gondii*, we sought to
263 undertake a more direct interrogation of the functionality of various ETC components upon

264 *Tg*QCR11 knockdown. To do this, we established an XFe96 flux analyzer-based assay that enabled
265 us to measure substrate-dependent mOCR using digitonin-permeabilized parasites. Permeabilizing
266 the plasma membrane of parasites with digitonin allows the passage of substrates with polar or
267 hydrophobic functional groups into parasites, where they can feed electrons into the ETC either
268 directly or via mitochondrial metabolism. Supplying different combinations of substrates and
269 inhibitors during the assay enables an assessment of the functionality of different ETC complexes
270 (Fig. 5A [26]).

271 We grew WT, *rTg*QCR11 and *rTg*ApiCox25 (an ATc-regulatable strain that enables knockdown
272 of the Complex IV protein *Tg*ApiCox25 [15]) parasites in the absence of ATc or in the presence
273 of ATc for 1-3 days. Parasites were starved for 1 hour in base media to deplete endogenous
274 substrates, and then permeabilized with 0.002% (w/v) digitonin. The five readings taken before
275 injection of substrate show that permeabilized parasites have very low OCR (Fig. 5B), indicating
276 that the 1 hour starvation successfully depleted endogenous substrates. Injection of the
277 tricarboxylic acid (TCA) cycle substrates malate and glutamate caused an almost instantaneous
278 increase in OCR in WT, *rTg*QCR11 and *rTg*ApiCox25 parasites that were cultured in the absence
279 of ATc (Fig. 5B). As this OCR could be abolished by subsequent injection of the Complex III
280 inhibitors antimycin A and atovaquone (Fig. 5B), these data indicate that the ETC is functional in
281 these parasites. The malate/glutamate-elicited mOCR of WT parasites grown for 3 days in the
282 presence of ATc was not significantly different to WT parasites grown in the absence of ATc (Fig.
283 5C), indicating that ATc itself does not impact ETC function. By contrast, knockdown of
284 *Tg*QCR11 and *Tg*ApiCox25 caused significant decreases in malate/glutamate-dependent mOCR
285 (Fig. 5C).

286 We next asked whether defects in malate/glutamate-dependent mOCR upon *TgQCR11* or
287 *TgApiCox25* knockdown occurred upstream or downstream of cytochrome *c*. To test this, we
288 injected the cytochrome *c* substrate N,N,N',N'-tetramethyl-p-phenylenediamine dihydrochloride
289 (TMPD). Reduced TMPD donates electrons directly to cytochrome *c*, downstream of Complex III,
290 and should therefore rescue mOCR in parasites with an ETC defect upstream of cytochrome *c* (Fig.
291 5A). By contrast, a Complex IV defect should not be rescued by TMPD since Complex IV is
292 downstream of cytochrome *c*. Injection of TMPD caused an increase in mOCR in *rTgQCR11*
293 parasites cultured in the presence of ATc (Fig. 5B). By contrast, *rTgApiCox25* parasites cultured
294 for 2 or 3 days on ATc had very little TMPD-dependent mOCR (Fig. 5B). Calculating the fold
295 stimulation of mOCR by TMPD relative to malate/glutamate-dependent mOCR indicates that
296 *rTgQCR11* parasites grown for 3 days on ATc have 10-fold greater stimulation of mOCR by
297 TMPD relative to malate (Fig. 5D). This demonstrates that Complex IV activity is largely retained
298 upon *TgQCR11* knockdown and implies that the defect in ETC activity upon loss of this protein
299 occurs upstream of cytochrome *c*. By contrast, *rTgApiCox25* parasites grown for 3 days on ATc
300 have little stimulation of mOCR by TMPD relative to malate/glutamate, indicating that loss of
301 *TgApiCox25* leads to a defect in Complex IV activity (Fig. 5D).

302 The preceding data indicate that loss of *TgQCR11* leads to defects in the ETC upstream of
303 cytochrome *c*. Since malate/glutamate can feed into the ETC via the TCA cycle [11, 27], it is
304 conceivable that defects we observe in mOCR upon *TgQCR11* knockdown could be the result of
305 defects in the TCA cycle rather than a selective defect in Complex III. To address this, we
306 measured mOCR using the substrate glycerol 3-phosphate, which donates electrons to CoQ
307 independently of the TCA cycle (Fig. 5A; [11, 27]). Knockdown of *TgQCR11* caused a similar
308 decrease in glycerol 3-phosphate-dependent mOCR compared to malate/glutamate-dependent

309 mOCR (Fig. 5E, F). Since mOCR elicited by two different substrates, one of which is independent
310 of the TCA cycle, was impaired by knockdown of *TgQCR11*, we conclude that loss of *TgQCR11*
311 likely leads to a selective defect in Complex III activity.

312 Given the severe defect in mOCR observed upon *TgQCR11* knockdown, we wondered whether
313 the integrity of Complex III is compromised by loss of *TgQCR11*. To assess this, we set out to
314 measure the effects of *TgQCR11* knockdown on several Complex III proteins. First, we integrated
315 TEV-HA tags into the 3' ends of the *TgQCR8* and *TgQCR12* open reading frames in the
316 *rTgQCR11*-FLAG parasite line (Fig. S10). We then grew *rTgQCR11*-FLAG/MPP α -HA,
317 *rTgQCR11*-FLAG/*TgQCR8*-HA and *rTgQCR11*-FLAG/*TgQCR12*-HA parasites in the absence of
318 ATc or in the presence of ATc for 1-3 days and assessed Complex III integrity by BN-PAGE.
319 Strikingly, the ~675 kDa complex of all three proteins was depleted upon *TgQCR11* knockdown
320 (Fig. 6A-C), suggesting that Complex III assembly and/or stability may be impaired upon loss of
321 *TgQCR11*. We wondered whether the abundance of Complex III proteins may also be affected by
322 *TgQCR11* knockdown, and assessed this by SDS-PAGE and western blotting. Interestingly, while
323 the abundance of MPP α remained consistent (Fig. 6D), the abundance of *TgQCR12* and *TgQCR8*
324 decreased by day 2-3 on ATc (Fig. 6E-F), suggesting that knockdown of *TgQCR11* decreases the
325 abundance of some Complex III proteins but not others. It is possible that MPP α abundance
326 remains unchanged because, in addition to being a component of Complex III, it exists in a lower
327 mass, ~220 kDa complex that our BN-PAGE analysis indicated is retained upon *TgQCR11*
328 knockdown (Fig. 6A), which could represent the mitochondrial processing peptidase in these
329 parasites. Together, these data indicate that *TgQCR11* is a novel, myzozoan-specific subunit of
330 Complex III in *T. gondii* that is critical for Complex III function by maintaining the integrity of
331 this protein complex.

332 Discussion

333 In this study, we characterized Complex III of the mitochondrial ETC in *T. gondii* parasites, which
334 we demonstrate exists as a ~675 kDa complex comprising 11 protein subunits (Fig.1). Our data
335 are consistent with an independent, parallel study by MacLean and colleagues, who identified the
336 same 11 subunits in a broader proteomic analysis of mitochondrial respiratory chain complexes in
337 *T. gondii* (MacLean *et al.* BioRxiv, 2020). The number of subunits we identified in *T. gondii*
338 Complex III is similar to that reported for bovine [28-30], yeast [31] and plants [19, 32] complexes
339 (11, 10 and 10 subunits, respectively), and the mass is similar to the fully-assembled yeast complex
340 (670 kDa; [33]). The overall architecture of Complex III in eukaryotes is broadly conserved as a
341 homodimer, with one copy of each subunit per monomer [28-31, 34], and we would suspect that
342 this is also the case for *T. gondii*. The sum of the predicted masses of the 11 *T. gondii* Complex III
343 subunits is ~350 kDa (assuming *TgCytB* mass to be ~41 kDa [20]), giving a mass of ~700 kDa for
344 the Complex III homodimer, which is in the ballpark of the ~675 kDa we observed by BN-PAGE.
345 A caveat to this conclusion is that we observed different abundances in some Complex III subunits
346 (Fig. S7), raising the possibility that not all subunits exist in a strict 1:1 stoichiometry per monomer.
347 In our proteomic analysis of *T. gondii* Complex III, we identified numerous canonical subunits,
348 including the three electron transporting subunits (*TgRieske*, *TgCytB* and *TgCytC1*), the two core
349 proteins (*TgMPP α* and *TgMPP β*), and two known additional (or so-called ‘supernumerary’)
350 subunits, the ‘hinge’ protein *TgQCR6* and ‘14 kDa’ protein *TgQCR7*. Our analysis also identified
351 two highly divergent supernumerary subunits, *TgQCR8* and *TgQCR9*, which were only
352 identifiable after considering the structural features of these proteins. This indicates that, like
353 several supernumerary proteins in Complexes IV and V of *T. gondii* [14-16], the ~1.5 billion years

354 of evolution since the common ancestor of apicomplexans and other eukaryotes has resulted in
355 considerable divergence in the sequences of these ETC proteins.

356 Intriguingly, our analysis also identified two novel Complex III subunits (*TgQCR11* and
357 *TgQCR12*) that are restricted to organisms closely related to *T. gondii*. *TgQCR11* has homologues
358 in other myzozoans – a lineage which comprises apicomplexans, as well as their closest free-living
359 relatives, the chromerids and dinoflagellates – whereas *TgQCR12* homologs are restricted to
360 apicomplexans. These observations fit with an emerging narrative that mitochondria of myzozoans
361 have evolved numerous proteins and functions that are unique amongst eukaryotes [13, 15, 35,
362 36]. The reasons for these novelties are unclear, but it is conceivable that the evolution of proteins
363 like *TgQCR11* and *TgQCR12* were necessitated by evolutionary pressures for greater ETC
364 efficiency and/or improved mitochondrial energy generation in the marine (and later parasitic)
365 niches in which these organisms evolved. We cannot rule out the possibility that *TgQCR11* and
366 *TgQCR12* homologs do exist in other lineages of eukaryotes, but have diverged to the extent that
367 they can no longer be detected through even the most sophisticated homology-based searches. In
368 future, obtaining a Complex III structure from apicomplexans will help reconcile these possibilities
369 and shed further light on how the highly diverged and novel subunits identified in this study
370 contribute to Complex III function.

371 In other eukaryotes, the small supernumerary subunits of Complex III have no well-established
372 functions, but are hypothesized to contribute to complex stability [34]. We demonstrate that loss
373 of *TgQCR11* leads to severe defects in parasite proliferation and mitochondrial oxygen
374 consumption (Fig. 4), decreased abundance of other Complex III subunits, and the disappearance
375 of the ~675 kDa Complex III (Fig. 6). These observations are all consistent with *TgQCR11* playing
376 a critical role in maintaining Complex III integrity by mediating the assembly and/or the stability

377 of this complex, much like supernumerary Complex III subunits from other eukaryotes. The exact
378 role of *TgQCR11* in these processes, however, remains elusive. Future functional analyses of
379 *TgQCR11*, including an examination of its position within the structure of Complex III, may
380 address its actual role in the complex.

381 In this study, we have developed a powerful suite of assays to probe different stages and complexes
382 of the ETC in *T. gondii* parasites. These assays rely on feeding permeabilized parasites with
383 specific ETC substrates and inhibitors at set times during the assays. We used these assays to
384 demonstrate that knockdown of *TgQCR11* caused a specific defect in Complex III function (Fig.
385 5), since a) the mOCR elicited by two independent substrate combinations (malate/glutamate and
386 glycerol 3-phosphate) was decreased upon *TgQCR11* knockdown, and b) the fold stimulation of
387 TMPD-dependent mOCR compared to malate/glutamate-dependent mOCR was high. This is
388 consistent with *TgQCR11* functioning downstream of CoQ yet upstream of cytochrome *c* (i.e. in
389 Complex III). By contrast, the Complex IV protein *TgApiCox25* [15] had a low-fold stimulation of
390 TMPD-dependent mOCR compared to malate/glutamate-dependent mOCR, consistent with
391 Complex IV functioning downstream of cytochrome *c* (i.e. in Complex IV). In future, these assays
392 will enable an in-depth characterization of the function of ETC proteins and complexes, and
393 provide a detailed understanding of the contribution of mitochondrial (and broader parasite)
394 biosynthetic and metabolic pathways to the ETC and energy generation in these parasites. We also
395 note that these approaches lend themselves to drug screening approaches for pin-pointing the target
396 of ETC inhibitors in these parasites.

397 Our work highlights the divergence of mitochondrial ETC Complex III composition in
398 apicomplexan parasites, providing important insights into what sets this major drug target apart

399 from the equivalent complex in host species. Future studies can now build on our findings to reveal
400 how novel subunits of this complex contribute to Complex III function and druggability.

401 **Materials and methods**

402 *Host cell and parasite culture*

403 *T. gondii* tachyzoites were cultured in human foreskin fibroblasts (HFF), as previously described
404 [25, 37]. In paired anhydrotetracycline (ATc) knockdown experiments, ATc (0.5 µg/ml) or ethanol
405 (vehicle control) was added to the media on required days. Plaque assays were performed as
406 described previously [37], with 500 parasites added per flask and incubated for 7-8 days before
407 staining with crystal violet.

408 *Genetic modifications of T. gondii*

409 TATiΔ*ku80* strain parasites [38] were used as the parental cell line in this study. All genetically
410 modified parasites were cloned by flow cytometry before being characterized.

411 To incorporate a 3' hemagglutinin tag containing a tobacco etch virus protease cleavage site (TEV-
412 HA tag) into the loci of *TgMPPα* and *TgCox2a*, we generated a vector expressing a single guide
413 RNA (sgRNA) targeting the region around the stop codon of *TgMPPα* and used an existing
414 sgRNA-expression plasmid to target *TgCox2a* [15]. To generate the *TgMPPα*-targeting vector, we
415 modified the pSAG1::Cas9-U6::sgUPRT (Addgene plasmid # 54467; [39]) vector using Q5
416 mutagenesis (New England Biolabs) as described previously [39]. For site-directed mutagenesis,
417 we used the primers MPPα 3'rep CRISPR fwd and the Universal Reverse primer (Table S2). We
418 also amplified a TEV-HA tag containing 50 bp of flanking sequence either side of the *TgMPPα* or
419 *TgCox2a* stop codon, using the primers MPPα tag fwd and MPPα tag rvs or Cox2a 3' rep fwd and
420 Cox2a 3' rep rvs, with a TEV-HA tag template synthesized as a gBlock (IDT; Table S2). The
421 sgRNA expressing vectors, which also expressed GFP-tagged Cas9, were co-transfected into

422 TATi $\Delta ku80$ strain parasites along with the TEV-HA tags, with transfections performed as
423 described previously [37]. GFP-positive parasites were selected by flow cytometry and cloned 3
424 days following transfection, then screened for successful integration using the primers MPP α 3'
425 screen fwd and MPP α 3' screen rvs or Cox2a 3' screen fwd and Cox2a 3' screen rvs (Table S2;
426 Fig. S1).

427 To introduce 3' FLAG epitope tags into the loci of *TgQCR8*, *TgQCR9*, *TgQCR11* and *TgQCR12*,
428 we generated vectors expressing a sgRNA targeting the region around their stop codons. To do
429 this, we modified the pSAG1::Cas9-U6::sgUPRT vector using Q5 mutagenesis with gene specific
430 3'rep CRISPR fwd primers and the Universal Reverse primer (Table S2). We also amplified a
431 FLAG tag containing 50 bp of flanking sequence either side of the stop codon of each gene, using
432 gene specific fwd and rvs primers, with FLAG tag template synthesized as a gBlock (IDT; Table
433 S2). We co-transfected the plasmid and PCR product into *TgMPP α -HA* strain parasites [18] and
434 also into TATi/ $\Delta ku80$ strain parasites for *TgQCR11*, selected GFP positive parasites by flow
435 cytometry 3 days post-transfection, then screened for successful integration using gene specific
436 screening fwd and rvs primers (Table S2; Fig. S6; Fig. S8).

437 To introduce an ATc-regulated promoter into the *TgQCR11* locus, we generated a vector
438 expressing a sgRNA targeting the region around the start codon of *TgQCR11*. To do this, we
439 modified the vector pSAG1::Cas9-U6::sgUPRT using Q5 mutagenesis with the primers QCR11
440 5' CRISPR fwd and the universal reverse primer (Table S2). We also PCR amplified the ATc-
441 regulated promoter plus a 'spacer' region consisting of part of the *T. gondii* DHFR open reading
442 frame and 3' UTR using the pPR2-HA3 vector [40] as template and the primers QCR11 pro rep
443 fwd and QCR11 pro rep rvs (Table S2), which each contain 50 bp of sequence specific for the
444 *TgQCR11* locus. We co-transfected the plasmid and the ATc-regulatable promoter into *TgQCR11-*

445 FLAG strain parasites, selected GFP positive parasites by flow cytometry 3 days post-transfection,
446 then screened for successful integration of the ATc-regulatable promoter using the primers QCR11
447 5' screen fwd and QCR11 5' screen rvs (Table S2; Fig. S8).

448 To generate a cell line where *TgMPP* α is HA tagged in the r*TgQCR11*-FLAG strain, we introduced
449 a 3' HA tag to the locus of *TgMPP* α using the vector described earlier that expresses a sgRNA
450 targeting the region around the stop codon of *TgMPP* α . We also amplified a HA tag containing 50
451 bp of flanking sequence either side of the *TgMPP* α stop codon, using the primers MPP α tag fwd
452 and MPP α tag rvs, with HA tag template synthesized as a gBlock (IDT; Table S2). We co-
453 transfected the plasmid and PCR product into r*TgQCR11*-FLAG strain parasites, selected GFP
454 positive parasites by flow cytometry 3 days post-transfection, then screened for successful
455 integration using the primers MPP α 3' screen fwd and MPP α 3' screen rvs (Table S2; Fig. S8).

456 To generate a vector that constitutively expressed *TgQCR11* for complementing the r*TgQCR11*
457 mutant, we ordered a gene block encoding the *TgQCR11* open reading frame plus a Ty1 epitope
458 tag (IDT; Table S2) and performed PCR using the primers QCR11 comp fwd and universal Ty1
459 rvs (Table S2). We digested the resulting PCR product with *Bgl*III and *Xma*I and ligated this into
460 the *Bgl*III and *Xma*I sites of the vector pUDTG (Yi Xue and G.v.D., unpublished). The resulting
461 vector contains a pyrimethamine-resistance DHFR cassette, a UPRT flanking sequence for
462 integration into the non-essential UPRT locus of *T. gondii*, and fuses a C-terminal Ty1 epitope tag
463 to the complementing *TgQCR11* protein. This vector was linearized in the UPRT flanking
464 sequence with *Mfe*I, transfected into r*TgQCR11*-FLAG/*TgMPP* α -HA parasites, and selected on
465 pyrimethamine (Sigma) as described [37].

466 To generate cell lines expressing either *TgQCR8*-TEV-HA or *TgQCR12*-TEV-HA in the
467 *rTgQCR11*-FLAG parasite strain, we introduced 3' TEV-HA tags into the loci of *TgQCR8* and
468 *TgQCR12*. We replicated the strategy for introducing FLAG tags into these loci that is described
469 above, but instead co-transfected the sgRNA-expressing vectors with TEV-HA tags. We amplified
470 TEV-HA tags containing 50 bp of flanking sequence either side of the stop codons using gene
471 specific fwd and rvs primers, with TEV-HA tag template synthesized as a gBlock (IDT; Table S2).
472 Following transfection, we selected GFP positive parasites by flow cytometry, then screened for
473 successful integration using gene specific screening fwd and rvs primers (Table S2; Fig. S10).

474 ***SDS-PAGE, BN-PAGE and immunoblotting***

475 Sodium dodecylsulfate (SDS)-polyacrylamide electrophoresis (PAGE), blue native (BN)-PAGE
476 and immunoblotting were performed as described previously [18, 41]. Primary antibodies used
477 included mouse anti-FLAG (1:100-1:2,000 dilution; Sigma clone M2), rat anti-HA (1:2000
478 dilution; Sigma clone 3F10), and rabbit anti-Tom40 (1:2,000 dilution; [18]). The secondary
479 antibodies used were horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Abcam),
480 goat anti-rabbit IgG (Abcam) and goat anti-rat IgG (Abcam). For probing for mouse antibodies on
481 immunoprecipitation western blots, HRP-conjugated anti-mouse TrueBlot ULTRA antibodies
482 (eBioscience) were used at 1:2,500 dilution. Blots were imaged using X-ray film or using a
483 ChemiDoc MP imaging system (BioRad).

484 ***Immunoprecipitation and mass spectrometry***

485 Immunoprecipitations were performed as described previously [18], except that parasite samples
486 were solubilized in 1% (v/v) Triton X-100. HA-tagged proteins were purified using anti-HA
487 affinity matrix (Sigma; rat anti-HA clone 3F10 antibodies) and FLAG-tagged proteins were

488 purified using anti-FLAG M2 affinity gel (Sigma; mouse anti-FLAG clone M2 antibodies). For
489 mass spectrometry sample preparation, parasite samples were solubilized in 1% (w/v) DDM, and
490 processed as described previously [15]. Briefly, anti-HA affinity matrix bound with HA-tagged
491 protein complexes were frozen at -80°C for 1 hr, then eluted at room temperature in 0.2 M glycine
492 containing 1% (w/v) DDM (pH 2.3). Samples were neutralized in ammonium bicarbonate, then
493 extracted in chloroform:methanol as described [42]. After extraction, the pellets were dried and
494 stored at -80°C before mass spectrometry analysis.

495 Mass spectrometry was conducted as previously described [15]. Briefly, samples were
496 resuspended in 8M Urea, 50 mM Tris pH 8.3 followed by reduction and alkylation. Solubilized
497 proteins were submitted to trypsin digestion overnight and the resulting peptides purified using the
498 C18 stage tips procedure. Peptides were reconstituted in 0.1% formic acid and 2% acetonitrile,
499 loaded onto a trap column and washed for 6 min before switching the precolumn in line with the
500 analytical column. The separation of peptides was performed as previously described (11). Data
501 were collected on a Q Exactive HF Orbitrap mass spectrometer (Thermo-Fisher Scientific) in Data
502 Dependent Acquisition mode using m/z 350–1500 as MS scan range at 60 000 resolution, HCD
503 MS/MS spectra were collected for the 10 most intense ions per MS scan at 15 000 resolution with
504 a normalized collision energy of 28% and an isolation window of 1.4 m/z. Dynamic exclusion
505 parameters were set as follows: exclude isotope on, duration 30 s and peptide match preferred.
506 Other instrument parameters for the Orbitrap were MS maximum injection time 30 ms with AGC
507 target 3×10^6 , MSMS for a maximum injection time of 110 ms with AGT target of 1.1×10^4 . The
508 raw data were uploaded into Peaks Studio 10.5 (Bioinformatics Solution Inc., Waterloo, Canada)
509 and processed with de novo peptide sequencing and Peaks DB using the ToxoDB
510 (<https://toxodb.org/toxo/>) database together with common contaminants (cRAP). For peptides

511 identification, the default settings were with precursor-ion and product-ion tolerances set to 10
512 ppm and 0.02 Da, respectively. Semispecific trypsin digest with a maximum of 1 missed cleavage
513 was employed and peptides were searched with carbamidomethylation of cysteine set as fixed
514 modification. To limit false-positive peptide identification, the false discovery rate (FDR) applied
515 to peptide-spectrum match (PSM) was set to 1%, and at least 1 unique peptide per protein was
516 used.

517 ***Immunofluorescence assays and microscopy***

518 Immunofluorescence assays were performed as described previously [41]. Primary antibodies used
519 were mouse anti-FLAG (1:500 dilution; Sigma clone M2), rat anti-HA (1:500 dilution; Sigma
520 clone 3F10), and rabbit anti-Tom40 (1:2,000 dilution; [18]). Secondary antibodies used were goat
521 anti-mouse Alexa Fluor 488 (1:500 dilution; Invitrogen), goat anti-rat Alexa Fluor 488 (1:500
522 dilution; Invitrogen), and goat anti-rabbit Alexa Fluor 546 (1:500 dilution; Invitrogen). Images
523 were acquired on a DeltaVision Elite deconvolution microscope (GE Healthcare) fitted with a
524 100X UPlanSApo oil immersion objective lens (NA 1.40). Images were deconvolved and adjusted
525 for contrast and brightness using SoftWoRx Suite 2.0 software, and subsequently processed using
526 Adobe Illustrator.

527 ***Seahorse XFe96 extracellular flux analysis***

528 Experiments measuring the oxygen consumption rate (OCR) and extracellular acidification rate
529 (ECAR) of intact extracellular parasites were conducted as described previously [15, 25]. We also
530 developed experiments to assess the OCR in digitonin-permeabilized parasites utilizing specific
531 ETC substrates. Briefly, parasites were harvested as for the XFe96 assays on intact parasites.
532 Parasites were then washed once in base medium (Agilent Technologies), resuspended in base

533 medium to 1.5×10^7 cells/mL and starved for 1 hour at 37°C to deplete endogenous ETC
534 substrates. 1.5×10^6 parasites were added to wells of Cell-Tak-coated Seahorse XFe96 cell culture
535 plates and adhered to the bottom by centrifugation ($800 \times g$, 3 min). Base medium was removed
536 and replaced with 175 μ L mitochondrial assay solution (MAS) buffer (220 mM mannitol, 70 mM
537 sucrose, 10 mM KH_2PO_4 , 5 mM MgCl_2 , 0.2% w/v fatty acid-free bovine serum albumin, 1 mM
538 EGTA, and 2 mM HEPES-KOH pH 7.4) containing 0.002 % (w/v) digitonin. ETC substrates and
539 inhibitors were loaded into Seahorse XFe96 sensor cartridge ports A-D, and injected into wells
540 during the experiment. OCR measurements were obtained every 3 min for five repeats before and
541 after injection of compounds (prepared in MAS buffer; concentrations given are final
542 concentrations following injection). Port A: FCCP (1 μ M) plus substrates. The substrates used
543 were malate plus glutamate (10 mM each) or sn-glycerol 3-phosphate bis(cyclohexylammonium)
544 salt (G3P; 25 mM). Port B: antimycin A and atovaquone (10 μ M and 1 μ M, respectively). Port C:
545 N,N,N',N'-tetramethyl-p-phenylenediamine dihydrochloride (TMPD; 0.2 mM) mixed with
546 ascorbic acid (3.3 mM). Port D: sodium azide (NaN_3 ; 10 mM). Substrate-elicited mOCR was
547 calculated by subtracting the non-mitochondrial OCR (the values following antimycin A and
548 atovaquone injection via Port B) from the OCR value obtained after substrate injection (Port A).
549 Likewise, TMPD-elicited mOCR was calculated by subtracting the non-mitochondrial OCR from
550 the OCR value obtained after TMPD injection (Port C). A minimum of 4 background wells were
551 used in each plate, and 3 technical replicates were used for each condition.

552 ***Data analyses and availability***

553 Data from the Seahorse flux analysis were exported from the Seahorse Wave Desktop software
554 (Agilent Technologies). A linear mixed effects model was applied to the data as described
555 previously [15], setting the error between plates (between experiments) and wells (within

556 experiments) as random effects, and the mOCR or ECAR values between cell lines and days on
557 drug (ATc) as fixed effects. Analysis of the least square means of the values was performed in the
558 R software environment. Statistical differences between these values were tested through ANOVA
559 (linear mixed effects), with a post hoc Tukey test.

560 Data from the *TgMPP α* -TEV-HA and *TgCox2a*-TEV-HA mass spectrometry-based proteomic
561 experiment were analyzed in the R software environment using the EdgeR package as described
562 previously [15, 43]. In the analysis performed to produce the volcano plot, only proteins identified
563 in both data sets and each biological replicate were included, while subsequent analyses also
564 considered proteins that were absent from one or more replicates of the *TgCox2a*-TEV-HA control.
565 The mass spectrometry proteomics data have been deposited to the ProteomeXchange consortium
566 via the Pride partner repository [44] with the dataset identified PXD018781 and
567 10.6019/PXD018781.

568 ***Bioinformatic analyses***

569 Amino acid sequences of *T. gondii* Complex III proteins were accessed from ToxoDB
570 (www.toxodb.org). Initial identification of homologs in the apicomplexan parasites *P. falciparum*
571 and *C. parvum*, and the chromerids *V. brassicaformis* and *C. velia*, was performed through Basic
572 Local Alignment Search Tool (tBLASTn) searches of the EuPathDB Transcripts database
573 (www.eupathdb.org, [45]). Where simple BLAST searches did not identify homologs, additional
574 homology searches were performed using the iterative search tool JackHMMER
575 (www.ebi.ac.uk/Tools/hmmer/search/jackhammer) and the profile hidden Markov model based
576 search tool HHPRED (<https://toolkit.tuebingen.mpg.de/tools/hhpred>, [23]). To identify homologs
577 of *TgQCR8* and *TgQCR9* in yeast, humans, *Arabidopsis* and the parasitic dinoflagellate *Perkinsus*

578 *marinus*, we performed JackHMMER and NCBI iterative PSI-BLAST [46] searches. We
579 identified dinoflagellate homologs of *TgQCR8*, *TgQCR9* and *TgQCR11* using BLAST searches
580 of *Symbiodinium* spp. genomes available at <http://reefgenomics.org> [47]. Transmembrane domain
581 predictions were performed using TMHMM [48] and TMPred ([https://embnet.vital-](https://embnet.vital-it.ch/software/TMPRED_form.html)
582 [it.ch/software/TMPRED_form.html](https://embnet.vital-it.ch/software/TMPRED_form.html)). Multiple protein sequence alignments of QCR8 (Fig. S2),
583 QCR9 (Fig. S3), *TgQCR11* (Fig. S4) and *TgQCR12* (Fig. S5) were performed using Clustal Omega
584 [49], and the graphical output was generated in BoxShade ([https://embnet.vital-](https://embnet.vital-it.ch/software/BOX_form.html)
585 [it.ch/software/BOX_form.html](https://embnet.vital-it.ch/software/BOX_form.html)). All accession numbers are included in the supplementary figure
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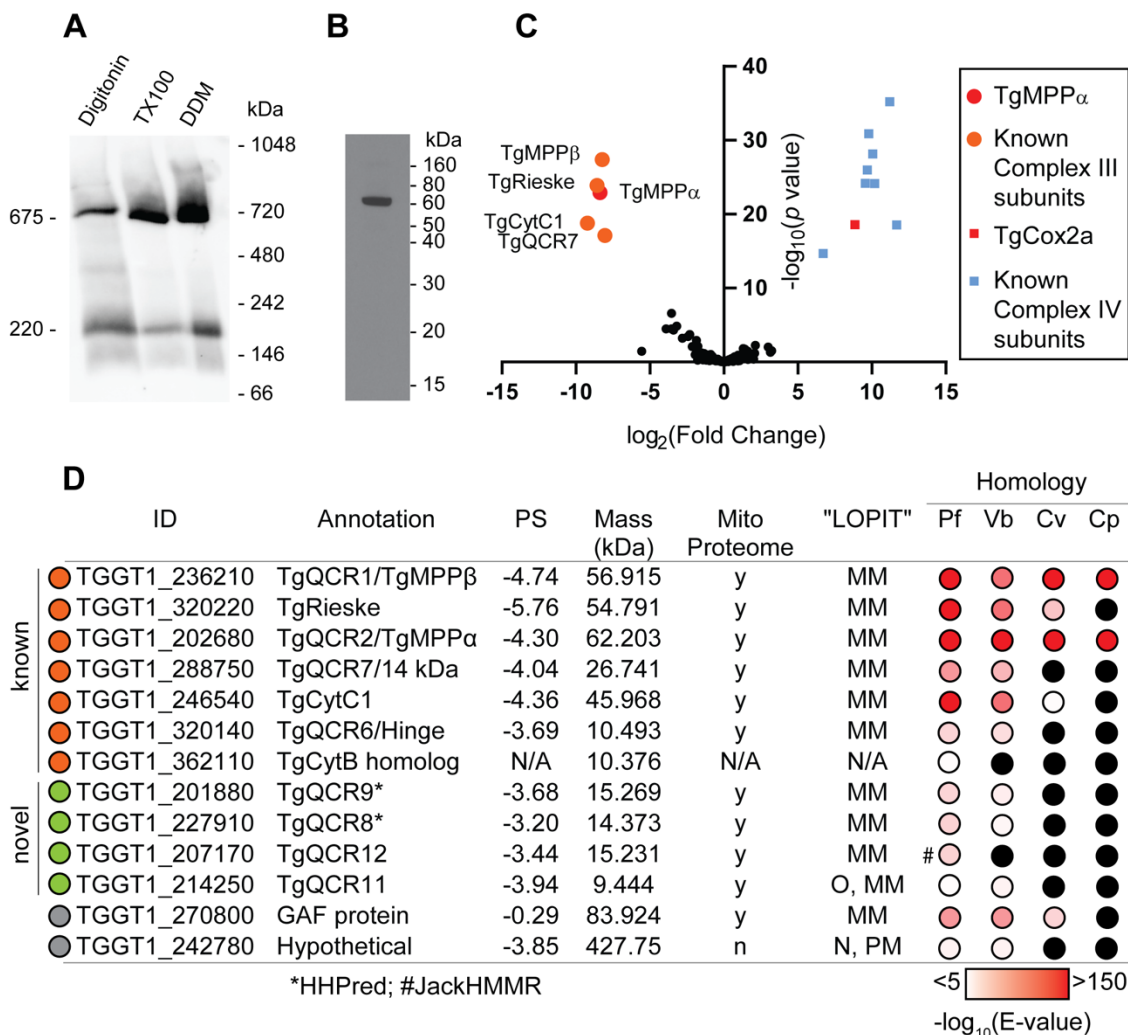
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- 750



751

752 **Fig 1: *TgMPPα* is part of a ~675 kDa protein complex and co-purifies with known**

753 **components of Complex III. (A)** Western blot of proteins extracted from *TgMPPα*-TEV-HA

754 parasites in 1% (w/v) digitonin, 1% (v/v) TX100 or 1% (w/v) DDM-containing lysis buffer,

755 separated by BN-PAGE, and detected with anti-HA antibodies. **(B)** Western blot of proteins

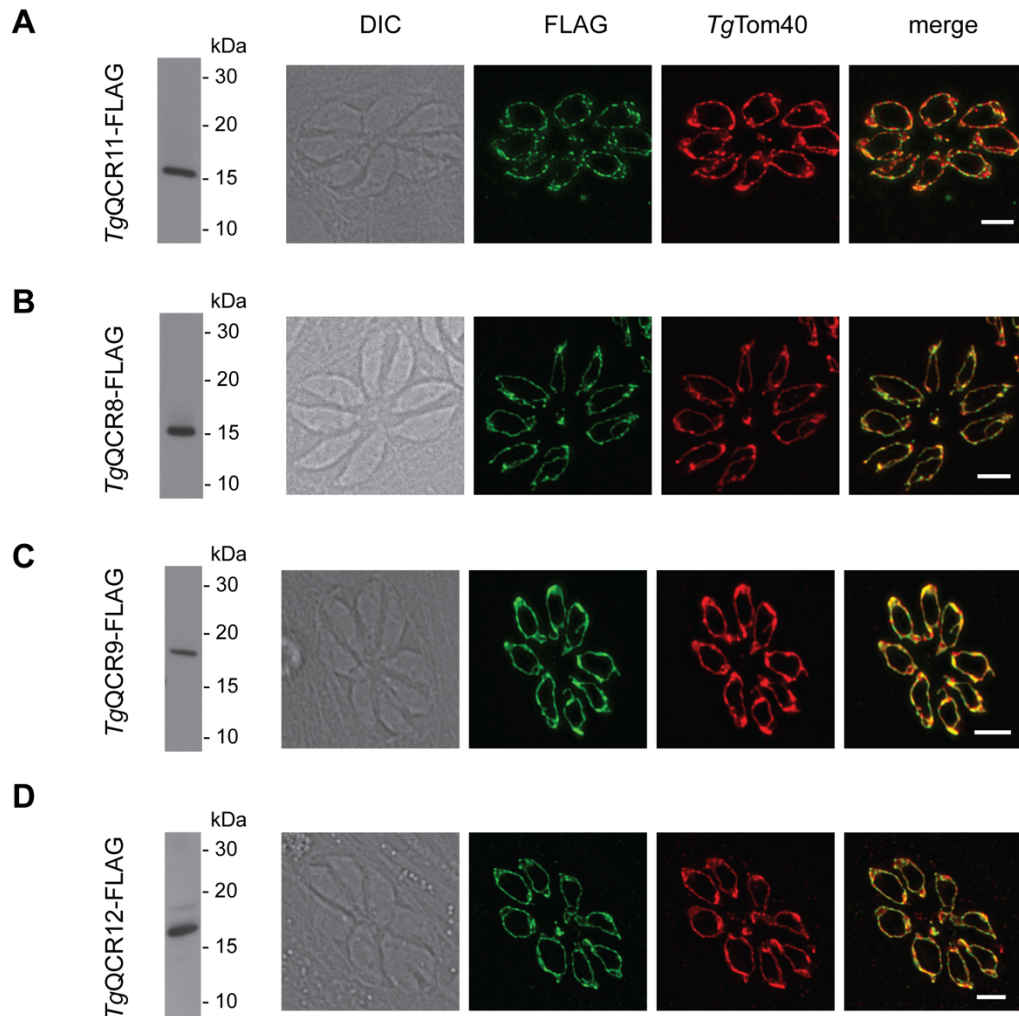
756 extracted from *TgMPPα*-TEV-HA parasites, separated by SDS-PAGE, and detected with anti-HA

757 antibodies. **(C)** Volcano plot showing the log₂ fold change vs -log₁₀ *p* values of proteins purified

758 from *TgMPPα*-TEV-HA vs *TgCox2a*-TEV-HA parasites using anti-HA immunoprecipitations and

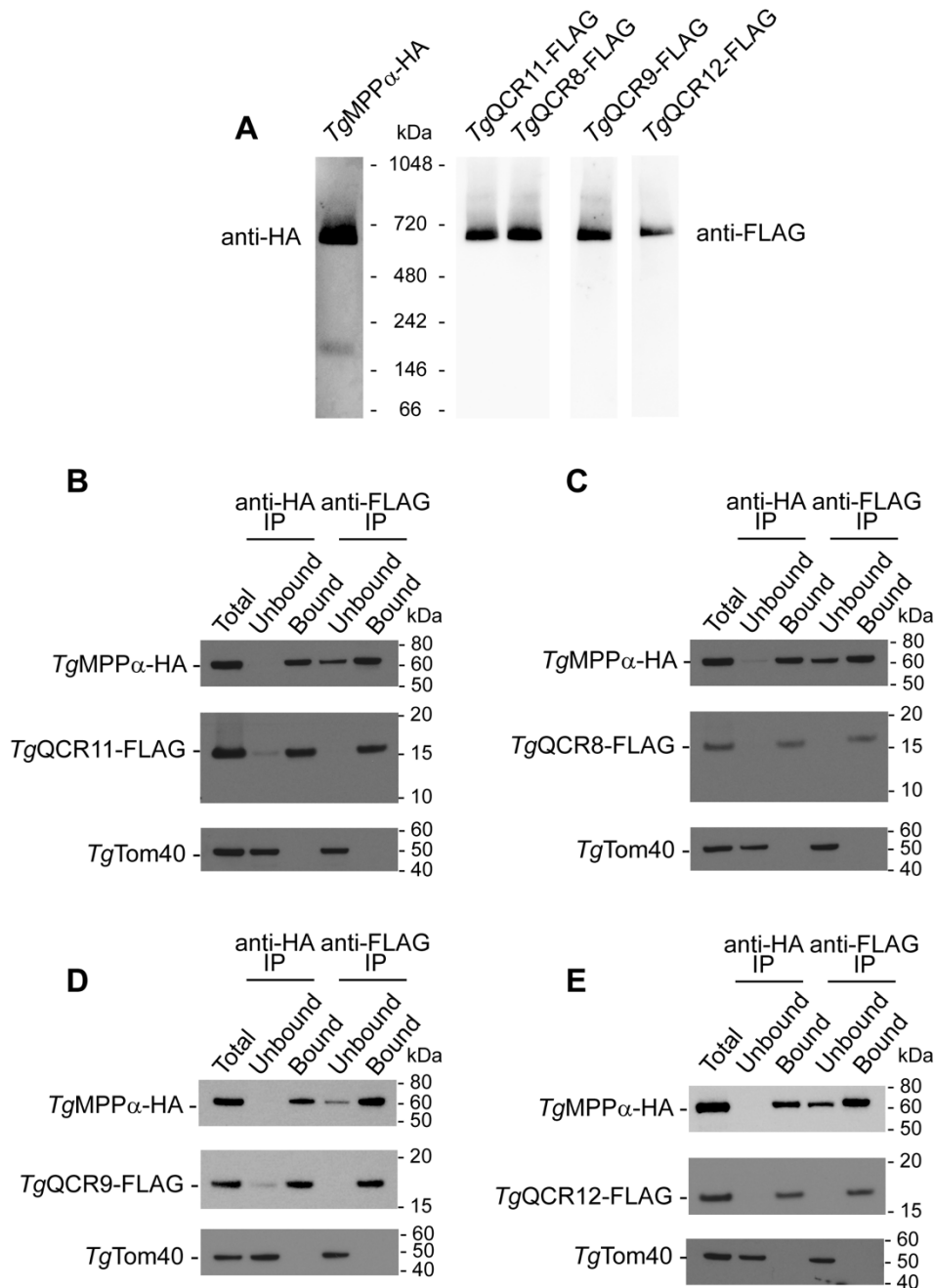
759 detected by mass spectrometry. To enable statistical comparisons, only proteins detected in all

760 three independent experiments for both parasite lines are depicted. Proteins enriched in the
761 *TgMPP* α -TEV-HA samples ($p < 0.05$; \log_2 fold change > 5) are labelled and shown in orange
762 circles, with *TgMPP* α shown in red. Similarly, proteins previously identified as *T. gondii* Complex
763 IV subunits [15] are shown in blue squares, with *TgCox2a* shown in red. **(D)** Table summarising
764 the characteristics of proteins identified in proteomic analysis of the *TgMPP* α -TEV-HA complex
765 (modelled after [16]). Proteins with homology to Complex III proteins are shown in orange circles,
766 novel or divergent Complex III proteins are depicted by green circles, and proteins identified in
767 the initial proteomic analysis but excluded in subsequent analyses are depicted in gray circles.
768 Protein IDs were obtained from ToxoDB and proposed annotations are listed. The phenotype score
769 (PS) of each gene predicts its importance for parasite proliferation, with scores < -2 typically found
770 in genes that are important for proliferation [24]. Detection in the mitochondrial proteome (Mito
771 Proteome, [15]) and its predicted cellular localisation (“LOPIT” [13]) are indicated (y = yes, n =
772 no, N/A = not available, MM = mitochondrial membranes, O = outlier, N = nucleus, PM = plasma
773 membrane). Homology indicates the tBLASTn expected value (E-value) between each *T. gondii*
774 protein sequence and its closest match in *Plasmodium falciparum* (Pf), *Vitrella brassicaformis*
775 (Vb), *Chromera velia* (Cv) or *Cryptosporidium parvum* (Cp) using EuPathDB searches. Black
776 circles indicate a close match could not be identified. *, homology detected using HHPRED; #,
777 homology detected using iterative JackHMMER searches.



778

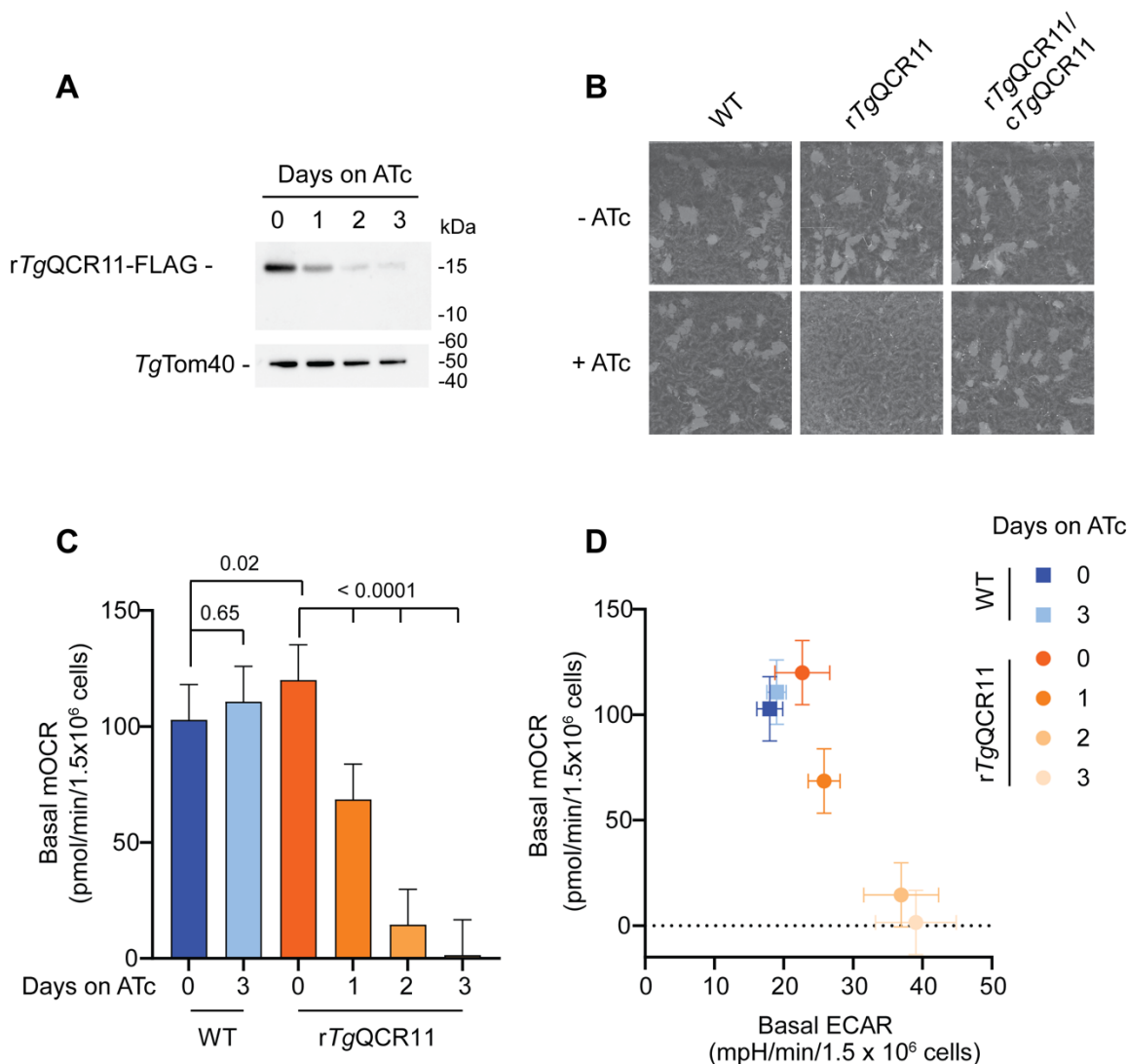
779 **Fig 2: Candidate Complex III subunits localize to the mitochondrion of *T. gondii*.** (Left)
780 Western blot and (Right) immunofluorescence assay analysis of (A) *TgMPP* α -HA/*TgQCR11*-
781 FLAG, (B) *TgMPP* α -HA/*TgQCR8*-FLAG, (C) *TgMPP* α -HA/*TgQCR9*-FLAG, or (D) *TgMPP* α -
782 HA/*TgQCR12*-FLAG parasites. Western blots were detected with anti-FLAG antibodies, and
783 immunofluorescence assays were detected with anti-FLAG (green) and the mitochondrial marker
784 anti-*TgTom40* (red) antibodies. Scale bars represent 2 μ m.



785

786 **Fig 3: Candidate Complex III subunits are part of a ~675 kDa protein complex and interact**
 787 **with TgMPP α .** (A) Western blot of proteins extracted from TgMPP α -TEV-HA (left) or TgMPP α -
 788 HA/TgQCR11-FLAG, TgMPP α -HA/TgQCR8-FLAG, TgMPP α -HA/TgQCR9-FLAG and
 789 TgMPP α -HA/TgQCR12-FLAG parasites in 1% (w/v) DDM, separated by BN-PAGE, and
 790 detected with anti-HA or anti-FLAG antibodies. Images were obtained from proteins transferred

791 to a single membrane, with lanes cut as indicated and probed with different concentration of
792 antibodies. **(B-E)** Western blots of proteins extracted from **(B)** *TgMPP α -HA/TgQCR11-FLAG*,
793 **(C)** *TgMPP α -HA/TgQCR8-FLAG*, **(D)** *TgMPP α -HA/TgQCR9-FLAG* or **(E)** *TgMPP α -*
794 *HA/TgQCR12-FLAG* parasites, and subjected to immunoprecipitation using anti-HA (anti-HA IP)
795 or anti-FLAG (anti-FLAG IP) antibody-coupled beads. Extracts include samples before
796 immunoprecipitation (Total), samples that did not bind to the anti-HA or anti-FLAG beads
797 (Unbound), and samples that bound to the anti-HA or anti-FLAG beads (Bound). Samples were
798 separated by SDS-PAGE, and probed with anti-HA antibodies to detect *TgMPP α -HA*, anti-FLAG
799 to detect *TgQCRs*, and anti-*TgTom40* as a control to detect an unrelated mitochondrial protein.



800

801 **Fig 4: The apicomplexan-specific Complex III subunit *TgQCR11* is important for parasite**

802 **proliferation and mitochondrial oxygen consumption. (A)** Western blot of proteins extracted

803 from rTgQCR11-FLAG/*TgMPPα*-HA parasites grown in the absence of ATc, or in the presence

804 of ATc for 1-3 days, separated by SDS-PAGE, and detected using anti-FLAG and anti-*TgTom40*

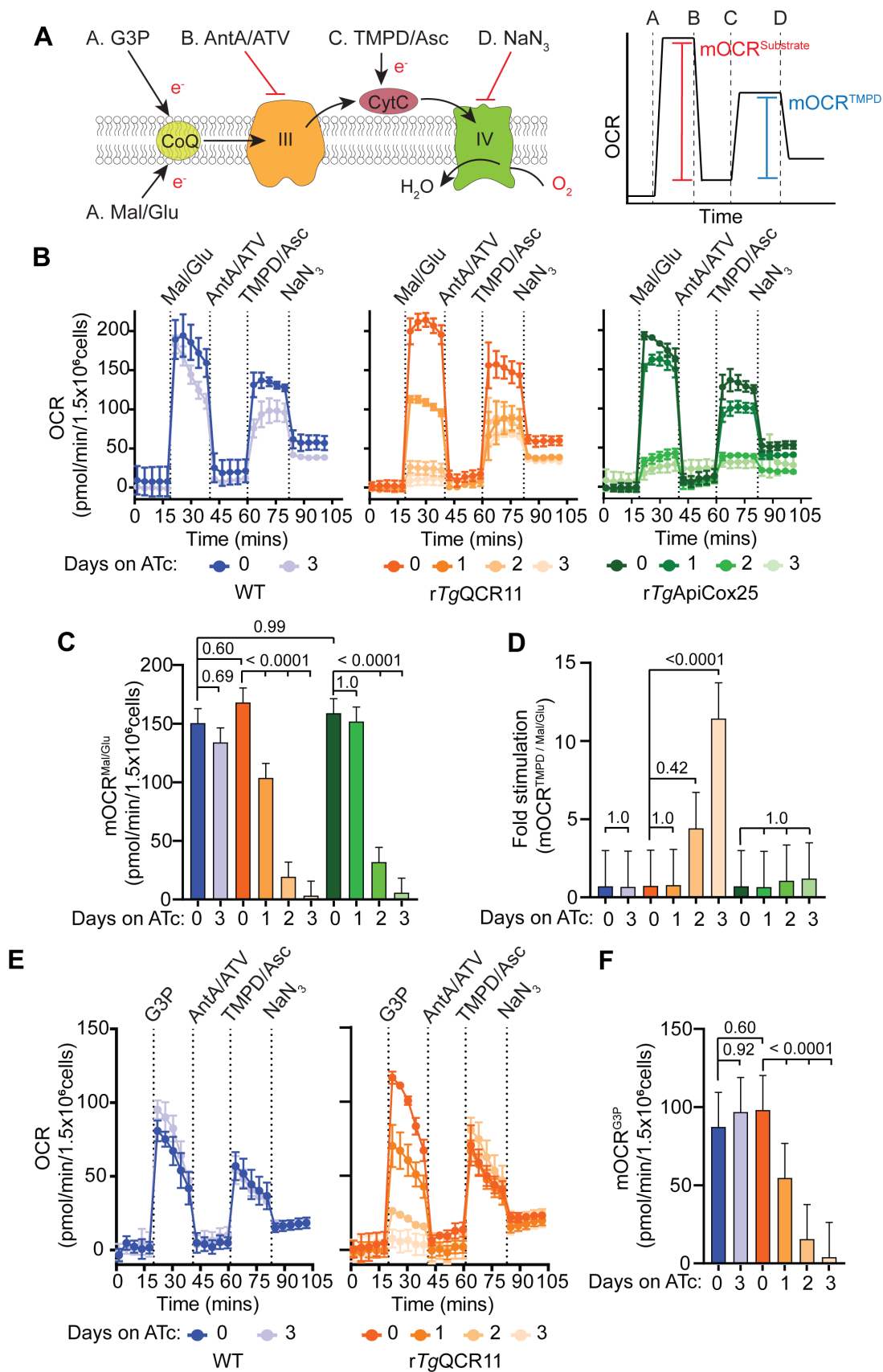
805 antibodies (loading control). **(B)** Plaque assays measuring growth of WT, rTgQCR11-

806 FLAG/*TgMPPα*-HA and complemented cTgQCR11-Ty1/rTgQCR11-FLAG/*TgMPPα*-HA

807 parasites cultured in the absence (top) or presence (bottom) of ATc for 8 days. Assays are from a

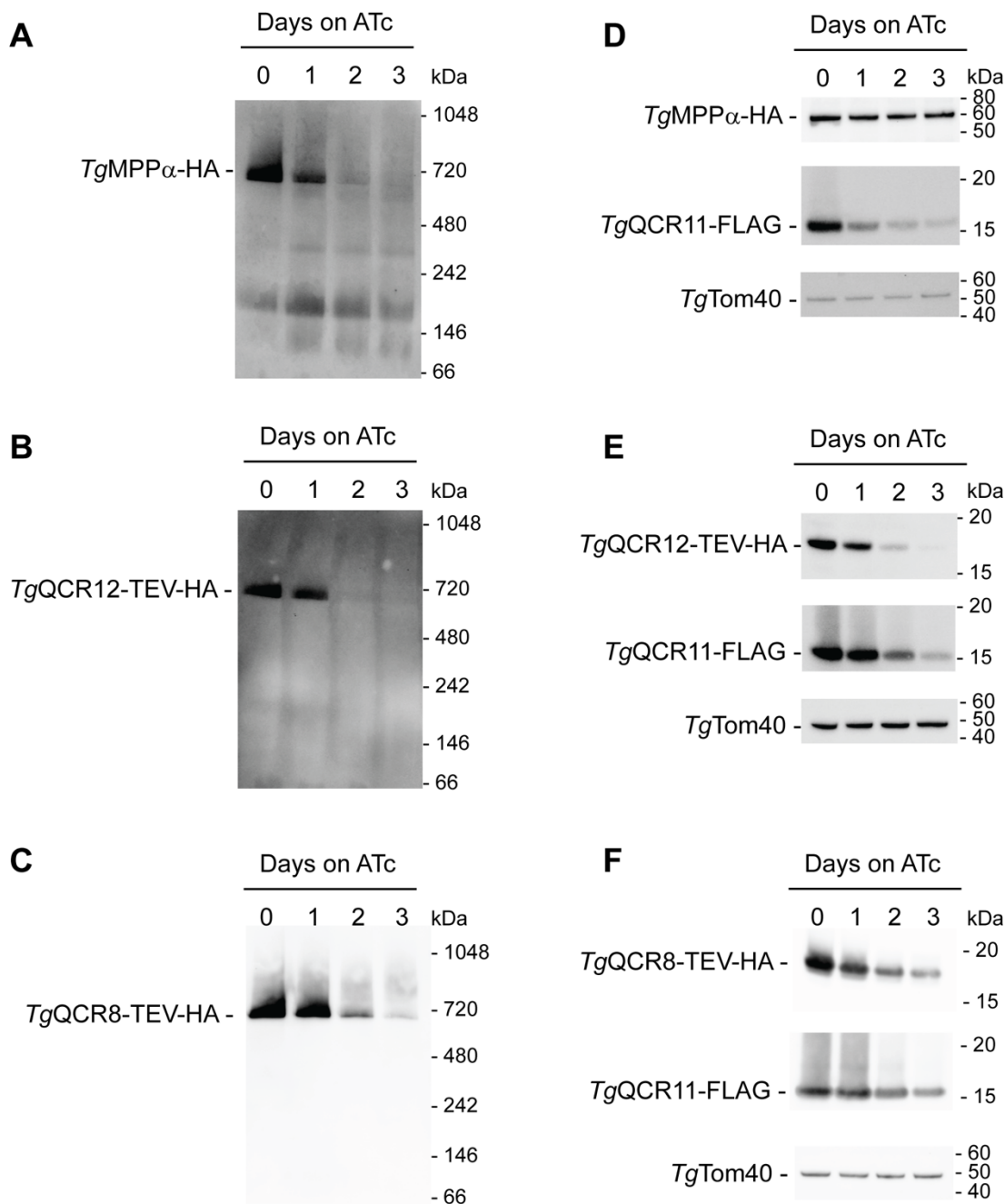
808 single experiment and are representative of 3 independent experiments. **(C)** Basal mitochondrial

809 oxygen consumption rates (mOCR) of extracellular WT parasites grown in the absence of ATc or
810 in the presence of ATc for 3 days (blue), and *rTgQCR11-FLAG/TgMPPA-HA* parasites grown in
811 the absence of ATc or in the presence of ATc for 1-3 days (orange). A linear mixed-effects model
812 was fitted to the data and values depict the least squares mean \pm 95% CI of three independent
813 experiments. ANOVA followed by Tukey's multiple pairwise comparisons test was performed,
814 with relevant *p* values shown. **(D)** Basal mOCR versus basal extracellular acidification rate
815 (ECAR) of WT parasites grown in the absence of ATc or in the presence of ATc for 3 days (blue),
816 and *rTgQCR11-FLAG/TgMPPA-HA* parasites grown in the absence of ATc or in the presence of
817 ATc for 1-3 days (orange). Data depict the mean mOCR and ECAR values \pm 95% CI of the linear
818 mixed-effects model ($n = 3$).



820 **Fig 5: Loss of *TgQCR11* leads to a specific defect in Complex III function.** (A) Schematic
821 diagram of the assay measuring OCR in extracellular digitonin-permeabilized parasites, with inset
822 (right) depicting a mock oxygen consumption rate (OCR) versus time graph to illustrate the typical
823 response of WT parasites. Parasites are starved for 1 hour in base media to deplete endogenous
824 energy sources, then permeabilized with 0.002% (w/v) digitonin before being subjected to the
825 following injections of substrates or inhibitors: Port A, malate and glutamate (Mal/Glu) or glycerol
826 3-phosphosphate (G3P); Port B, antimycin A and atovaquone (AntA/ATV); Port C, TMPD and
827 ascorbate (TMPD/Asc); Port D, sodium azide (NaN₃). The mitochondrial OCR (mOCR) elicited
828 by a substrate (red line, mOCR^{substrate}) and the mOCR elicited by TMPD/Asc (blue line,
829 mOCR^{TMPD}) are then calculated from these data. CytC, cytochrome *c*; CoQ, coenzyme Q; III,
830 Complex III; IV, Complex IV; e⁻, electrons. (B) Representative traces depicting OCR over time
831 when supplying Mal/Glu (10 mM) as an energy source. WT (blue), *rTgQCR11-FLAG/TgMPPα*-
832 HA (orange) and *rTgApiCox25-HA* (green) parasites were grown in the absence of ATc or in the
833 presence of ATc for 1-3 days. Data represent the mean ± SD of three technical replicates, and are
834 representative of three independent experiments. (C) Mal/Glu elicited mOCR (mOCR^{Mal/Glu}) of
835 WT (blue), *rTgQCR11-FLAG/TgMPPα*-HA (orange) and *rTgApiCox25-HA* (green) parasites that
836 were grown in the absence of ATc or in the presence of ATc for 1-3 days. A linear mixed-effects
837 model was fitted to the data and values depict the least squares mean ± 95% CI from three
838 independent experiments. ANOVA followed by Tukey's multiple pairwise comparisons test was
839 performed, with relevant *p* values shown. (D) Fold stimulation of mOCR by TMPD relative to
840 Mal/Glu in WT (blue), *rTgQCR11-FLAG/TgMPPα*-HA (orange) and *rTgApiCox25-HA* (green)
841 parasites that had been grown in the absence of ATc or in the presence of ATc for 1-3 days (mean
842 ± 95% CI of the linear mixed-effects model; n = 3). ANOVA followed by Tukey's multiple

843 pairwise comparisons test was performed, with relevant p values shown. **(E)** Representative traces
844 depicting OCR over time when supplying the TCA-independent substrate G3P (10 mM) as an
845 energy source. WT (blue) and *rTgQCR11-FLAG/TgMPP α -HA* (orange) parasites were grown in
846 the absence of ATc or in the presence of ATc for 1-3 days. Data represent the mean \pm SD of three
847 technical replicates, and are representative of three independent experiments. **(F)** G3P elicited
848 mOCR (mOCR^{G3P}) of WT (blue) and *rTgQCR11-FLAG/TgMPP α -HA* (orange) parasites that were
849 grown in the absence of ATc or in the presence of ATc for 1-3 days (mean \pm 95% CI of the linear
850 mixed-effects model; $n = 3$). ANOVA followed by Tukey's multiple pairwise comparisons test
851 was performed, with relevant p values shown.



852

853 **Fig 6: *TgQCR11* is important for Complex III integrity.** (A-C) Western blots of proteins
854 extracted from (A) r*TgQCR11*-FLAG/*TgMPP α* -HA, (B) r*TgQCR11*-FLAG/*TgQCR12*-TEV-HA,
855 and (C) r*TgQCR11*-FLAG/*TgQCR8*-TEV-HA parasites that had been grown in the absence of
856 ATc or in the presence of ATc for 1-3 days. Samples were prepared in 1% (w/v) DDM, separated

857 by BN-PAGE, and detected with anti-HA antibodies. **(D-F)** Western blots of proteins extracted
858 from **(D)** *rTgQCR11-FLAG/TgMPP α -HA*, **(E)** *rTgQCR11-FLAG/TgQCR12-TEV-HA*, and **(F)**
859 *rTgQCR11-FLAG/TgQCR8-TEV-HA* parasites that had been grown in the absence of ATc or in
860 the presence of ATc for 1-3 days. Samples were separated by SDS-PAGE, and probed with anti-
861 HA, anti-FLAG and anti-*TgTom40* (loading control) antibodies. Western blots shown are
862 representative of at least two independent experiments, with matched BN-PAGE and SDS-PAGE
863 samples prepared from the same experiment.