

1 **Evidence that disruption of apicoplast protein import in malaria parasites evades delayed-**  
2 **death growth inhibition**

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13 Running Title: Disruption of apicoplast protein import

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21 **Abstract**

22 Malaria parasites (*Plasmodium* spp.) contain a nonphotosynthetic plastid organelle called the  
23 apicoplast, which houses essential metabolic pathways and is required throughout the parasite  
24 life cycle. Hundreds of proteins are imported across 4 membranes into the apicoplast to support  
25 its function and biogenesis. The machinery that mediates this import process is distinct from  
26 proteins in the human host and may serve as ideal drug targets. However, a significant concern is  
27 whether inhibition of apicoplast protein import will result in a “delayed-death” phenotype that  
28 limits clinical use, as observed for inhibitors of apicoplast housekeeping pathways. To assess the  
29 growth inhibition kinetics of disrupting apicoplast protein import, we targeted a murine  
30 dihydrofolate reductase (mDHFR) domain, which is stabilized by the compound WR99210, to  
31 the apicoplast to enable inducible blocking of apicoplast-localized protein translocons. We show  
32 that stabilization of this apicoplast-targeted mDHFR disrupts parasite growth within a single lytic  
33 cycle in an apicoplast-specific manner. Consistent with inhibition of apicoplast protein import,  
34 stabilization of this fusion protein disrupted transit peptide processing of endogenous apicoplast  
35 proteins and caused defects in apicoplast biogenesis. These results indicate that disruption of  
36 apicoplast protein import avoids delayed-death growth inhibition and that target-based  
37 approaches to develop inhibitors of import machinery may yield viable next-generation  
38 antimalarials.

39 **Importance:** Malaria is a major cause of global childhood mortality. To sustain progress in  
40 disease control made in the last decade, new antimalarial therapies are needed to combat  
41 emerging drug resistance. Malaria parasites contain a relict chloroplast called the apicoplast,  
42 which harbors new targets for drug discovery, including import machinery that transports  
43 hundreds of critical proteins into the apicoplast. Unfortunately, some drugs targeting apicoplast

44 pathways show delayed growth inhibition, which results in a slow onset-of-action that precludes  
45 their use as fast-acting, frontline therapies. We used a chemical biology approach to disrupt  
46 apicoplast protein import and showed that chemical disruption of this pathway avoids delayed  
47 growth inhibition. Our finding indicates that prioritization of proteins involved in apicoplast  
48 protein import for target-based drug discovery efforts may aid in the development of novel fast-  
49 acting antimalarials.

50

## 51 **Introduction**

52 *Plasmodium* spp. parasites cause malaria and are responsible for over 200 million human  
53 infections and over 400,000 deaths annually (1). Despite a reduction in malaria-related mortality  
54 in the past 15 years, emerging resistance to frontline antimalarials necessitates the continued  
55 development of new chemotherapies (2, 3). One key source of drug targets is the apicoplast, a  
56 nonphotosynthetic plastid organelle found in many apicomplexan pathogens (4, 5). The  
57 apicoplast produces essential metabolites required for parasite replication (6). Apicoplast  
58 function requires the import of over 300 nuclear-encoded gene products into the organelle,  
59 including biosynthetic enzymes and pathways that support organelle biogenesis and maintenance  
60 (7). Most apicoplast proteins are 1) synthesized on cytosolic ribosomes, 2) trafficked to the  
61 apicoplast via the endoplasmic reticulum (ER), and 3) translocated across the 4 apicoplast  
62 membranes. This multistep import pathway involves more than a dozen proteins, including  
63 homologs of the translocation and ubiquitylation machinery typically involved in ER-associated  
64 degradation (ERAD) (8-12) as well as homologs of the TOC and TIC machinery found in plant  
65 plastids (9, 13-15).

66 Apicoplast protein import machinery are potential drug targets, but a key unresolved  
67 question is whether inhibition of apicoplast protein import causes a “delayed-death” phenotype  
68 that has been observed for inhibitors of apicoplast housekeeping functions (16, 17). During  
69 delayed death, parasite growth is unaffected during the first lytic cycle of inhibitor treatment but  
70 is severely inhibited in the second lytic cycle even after drug removal. This *in vitro* phenotype  
71 manifests as a slow onset-of-action that limits clinical use of these drugs. Unfortunately, there  
72 are no inhibitors known to act directly on apicoplast protein import, precluding direct assessment  
73 of growth inhibition kinetics. Furthermore, most genetic tools available in *Plasmodium* parasites  
74 act at the DNA or RNA levels (18), which can result in different growth inhibition kinetics than  
75 direct chemical inhibition of that same target (19-21). Destabilization domains that conditionally  
76 target proteins for degradation by the cytosolic ubiquitin-proteasome enable protein-level  
77 disruption (22, 23), but these systems are not suitable to study apicoplast-localized proteins,  
78 which are inaccessible to the cytosolic proteasome.

79 Given these limitations, we developed a protein-level tool to dissect growth inhibition  
80 kinetics following disruption of apicoplast protein import. A murine dihydrofolate reductase  
81 (mDHFR) domain which can be conditionally stabilized by a small molecule has been used to  
82 characterize protein translocation steps during yeast mitochondrial protein import *in vitro* (24)  
83 and, more recently, during export of malarial proteins across the parasitophorous vacuole (PV)  
84 membrane into the host red blood cell (25, 26). Strikingly, in the context of malarial protein  
85 export, mDHFR stabilized with the compound WR99210 can block the translocon to prevent  
86 export of other *Plasmodium* proteins and disrupt parasite growth (27). Given that there are at  
87 least three putative translocation steps during apicoplast protein import (7), we reasoned that we

88 could use a similar strategy to block apicoplast translocons and disrupt protein import in a  
89 manner that resembles inhibition with a small molecule (Fig. 1A).

90

## 91 **Results and Discussion**

92 To generate a protein-level conditional tool for disruption of apicoplast protein import, we  
93 targeted a GFP-mDHFR fusion to the apicoplast via the *N*-terminal leader sequence of acyl  
94 carrier protein (ACP) (Fig. 1B). As a negative control, we generated a cell line expressing the  
95 same fusion with a lysine 18 to glutamate (K18E) mutation in the ACP leader sequence that  
96 renders this transit peptide nonfunctional and causes mistargeting to the PV (28). Both GFP-  
97 mDHFR fusions were expressed in *P. falciparum* Dd2<sup>attB</sup> parasites (29) and localized to the  
98 expected compartments (Fig. 1C).

99 To test whether stabilization of these GFP-mDHFR fusions disrupted parasite growth, we  
100 treated ring-stage parasites with increasing doses of WR99210 and assessed parasitemia after 3  
101 days as a read-out for parasite growth inhibition during the first lytic cycle. Parental Dd2<sup>attB</sup>  
102 parasites, which express a WR99210-resistant human DHFR allele, were unaffected at the  
103 WR99210 concentrations tested (Fig. 2A). Parasites expressing the PV-localized ACP<sub>L</sub>(K18E)-  
104 GFP-mDHFR fusion from the *attB* site were also insensitive to WR99210. In contrast, parasites  
105 expressing the apicoplast-targeted ACP<sub>L</sub>-GFP-mDHFR fusion showed dose-dependent growth  
106 inhibition in response to WR99210 (Fig. 2A).

107 During *in vitro* culture of blood-stage *P. falciparum*, biosynthesis of the isoprenoid  
108 precursor isopentenyl pyrophosphate (IPP) is the only essential function of the apicoplast. As  
109 such, supplementation with exogenous IPP can rescue apicoplast defects and can be used to  
110 identify apicoplast-specific phenotypes (30). IPP supplementation reversed the WR99210

111 sensitivity of parasites expressing ACP<sub>L</sub>-GFP-mDHFR in both a 3-day dose-response experiment  
112 and over a 5-day time course (Fig. 2A and B). These results indicate that the WR99210  
113 sensitivity conferred by ACP<sub>L</sub>-GFP-mDHFR is due to disruption of an apicoplast-specific  
114 pathway.

115         Given our hypothesized mechanism-of-action inhibiting apicoplast protein import (Fig.  
116 1A), we expected that ACP<sub>L</sub>-GFP-mDHFR stabilization would cause global disruption of the  
117 apicoplast, including organelle maintenance and biogenesis, leading to apicoplast loss. To assess  
118 the status of the apicoplast, we assayed the presence of the apicoplast genome in WR99210-  
119 treated, IPP-rescued parasites by quantitative PCR (qPCR). WR99210 treatment caused a  
120 decrease in the apicoplast:nuclear genome ratio beginning at 1 day post-treatment and near-  
121 complete loss of the apicoplast genome after 2 lytic cycles (Fig. 3). The loss of the apicoplast  
122 genome, a key marker of the apicoplast, indicates that chemical stabilization of ACP<sub>L</sub>-GFP-  
123 mDHFR-expressing parasites caused apicoplast loss.

124         Sorting of apicoplast cargo occurs in the parasite endomembrane system, after which  
125 proteins are thought to traffic to the apicoplast by vesicle transport. While we expected that  
126 stabilization of ACP<sub>L</sub>-GFP-mDHFR would disrupt transit of apicoplast cargo across the  
127 apicoplast-localized ERAD- and TOC/TIC translocons, there was a possibility that this stabilized  
128 fusion protein could disrupt the upstream sorting process and prevent apicoplast proteins from  
129 ever reaching the organelle. Previous data suggest that defects in sorting apicoplast cargo  
130 manifest as mistargeting of apicoplast proteins to the PV (28). To assess whether stabilization of  
131 ACP<sub>L</sub>-GFP-mDHFR blocked protein import early during sorting or later after arrival at the  
132 apicoplast, we used live- and fixed-cell imaging to assess whether this fusion protein and the  
133 endogenous apicoplast protein ACP were successfully sorted in WR99210-treated parasites. As

134 expected, in untreated parasites ACP<sub>L</sub>-GFP-mDHFR localized to an elongated structure  
135 characteristic of the apicoplast and co-localized with ACP (Fig. 4A and B). After 1 day of  
136 WR99210 treatment (i.e., within the same lytic cycle as treatment), the majority of cells  
137 exhibited ACP<sub>L</sub>-GFP-mDHFR and ACP signal in a single punctum or elongated pattern that  
138 likely indicates an intact apicoplast (Fig. 4A and B). After 3 days of WR99210 treatment (i.e.,  
139 after 1 full lytic cycle of treatment), both ACP<sub>L</sub>-GFP-mDHFR and ACP were present almost  
140 exclusively in diffuse puncta that are thought to represent vesicles post-sorting in parasites that  
141 have lost their apicoplast (30) (Fig. 4A and B). Altogether, the localization of ACP<sub>L</sub>-GFP-  
142 mDHFR and ACP to either organelle-like structures or diffuse puncta following mDHFR  
143 stabilization indicates that sorting in the parasite endomembrane system remained intact. This  
144 suggests that the defect in WR99210-treated ACP<sub>L</sub>-GFP-mDHFR parasites occurs after this  
145 initial protein sorting step.

146       Most apicoplast-targeted proteins contain an *N*-terminal transit peptide that is  
147 proteolytically processed upon successful import into the organelle lumen, and an accumulation  
148 of unprocessed protein can be used as a marker for a protein import defect. We expected that  
149 stabilized ACP<sub>L</sub>-GFP-mDHFR would block translocation of apicoplast cargo into the organelle  
150 lumen and cause a transit peptide processing defect in WR99210-treated parasites. We therefore  
151 assessed the processing of the ACP<sub>L</sub>-GFP-mDHFR fusion and the endogenous apicoplast protein  
152 ClpP by western blotting. Consistent with a defect in protein translocation, WR99210-treated  
153 parasites exhibited a modest but reproducible accumulation of unprocessed ACP<sub>L</sub>-GFP-mDHFR  
154 after 1 day of WR99210 treatment (Fig. 4C and D). ClpP showed a comparable transit peptide  
155 processing defect (Fig. 4C and D), suggesting that stabilization of ACP<sub>L</sub>-GFP-mDHFR affected  
156 import not only of the fusion protein itself but also of endogenous apicoplast cargo. Processing

157 of both the GFP-mDHFR fusion and ClpP was almost completely ablated after 3 days of  
158 WR99210 treatment (Fig. 4C and D). Because apicoplast proteins are still sorted to the  
159 apicoplast (Fig. 4A and B) but show a transit peptide processing defect (Fig. 4C and D), these  
160 data are consistent with a disruption in the translocation of apicoplast proteins across one or  
161 more apicoplast membranes.

162 In lieu of direct inhibitors of apicoplast protein import, we used a chemical biology  
163 approach to conditionally block apicoplast protein import by addition of a small molecule.  
164 Altogether, our results suggest a model in which chemically stabilized ACP<sub>L</sub>-GFP-mDHFR  
165 traffics to the apicoplast and stalls within putative membrane translocons, preventing import of  
166 endogenous apicoplast cargo and disrupting apicoplast biogenesis and function. Given that  
167 apicoplast protein import is likely required for biogenesis pathways such as apicoplast genome  
168 replication, the emergence of both a transit peptide processing defect (a readout for protein  
169 import; Fig. 4C and D) and a genome replication defect (Fig. 3) after 1 day of ACP<sub>L</sub>-GFP-  
170 mDHFR stabilization are consistent with this model. Unfortunately, because we could not detect  
171 direct biochemical interaction of stabilized ACP<sub>L</sub>-GFP-mDHFR with apicoplast translocons, we  
172 cannot rule out translocation-independent mechanisms-of-action, such as toxicity due to  
173 accumulation of stably folded ACP<sub>L</sub>-GFP-mDHFR in the apicoplast lumen. However, given the  
174 previous uses of mDHFR fusions to block protein translocation (24-27) and the defect in  
175 apicoplast protein import observed after just 1 day of WR99210 treatment (Fig. 4C and D), our  
176 model seems the most parsimonious.

177 These findings suggest that the proteins required for apicoplast protein import can serve  
178 as antimalarial targets that avoid delayed-death growth inhibition. Over a dozen proteins have  
179 been implicated in import of nuclear-encoded apicoplast proteins, but of particular interest are



180 proteins related to established drug targets in other systems. For example, the apicoplast-  
181 localized AAA ATPase CDC48 is related to mammalian p97 and is likely involved in  
182 translocation of apicoplast cargo across the periplastid membrane (12). Notably, mammalian  
183 p97, which plays an important role in ERAD and other cellular processes, is of interest as an  
184 anti-cancer drug target. Specific inhibitors of mammalian p97 have been developed (31-34),  
185 suggesting that the same could be accomplished for the apicoplast-localized CDC48 in  
186 apicomplexans. Similarly, components of an apicoplast-localized ubiquitylation system are  
187 essential for apicoplast protein import and may also represent potential drug targets (11). A small  
188 molecule inhibitor of UAE, a ubiquitin-activating enzyme in humans, has recently been reported  
189 to have activity in multiple tumor models (35), indicating that the apicoplast-localized ubiquitin  
190 activating enzyme may also be a valuable drug target. The druggability of other members of  
191 apicoplast protein import complexes has not been explored and may yield additional targets.  
192 Overall, our data suggest that chemical inhibition of apicoplast protein import machinery may be  
193 a viable strategy for development of next-generation antimalarials. Target-based drug discovery  
194 efforts against known import machinery may therefore yield specific inhibitors of apicoplast  
195 biogenesis with mechanisms-of-action orthogonal to those of current antimalarials.

196

## 197 **Materials and Methods**

### 198 **Ethics statement**

199 Human erythrocytes were purchased from the Stanford Blood Center (Stanford, California) to  
200 support in vitro *P. falciparum* cultures. Because erythrocytes were collected from anonymized  
201 donors with no access to identifying information, IRB approval was not required. All consent to  
202 participate in research was collected by the Stanford Blood Center.

203

## 204 **Parasite growth**

205 *P. falciparum* Dd2<sup>attB</sup> parasites (MRA-843) were obtained from MR4 and were grown in human  
206 erythrocytes (2% hematocrit) obtained from the Stanford Blood Center in RPMI 1640 media  
207 (Gibco) supplemented with 0.25% AlbuMAX II (Gibco), 2 g/L sodium bicarbonate, 0.1 mM  
208 hypoxanthine (Sigma), 25 mM HEPES, pH 7.4 (Sigma), and 50 µg/L gentamicin (Gold  
209 Biotechnology) at 37°C, 5% O<sub>2</sub>, and 5% CO<sub>2</sub>.

210

## 211 **Vector construction**

212 Oligonucleotides (Table 1) were purchased from the Stanford Protein and Nucleic Acid facility  
213 and molecular cloning was performed using In-Fusion cloning (Clontech). GFP and mDHFR  
214 were PCR amplified from pARL2-SBP1-mDHFR-GFP (27) using primers MB119/MB120 and  
215 MB121/MB122, respectively. These products were simultaneously cloned into the BsiWi/AflII  
216 sites of the plasmid pRL2-ACP<sub>L</sub>-GFP (36) or a similar plasmid containing the ACP<sub>L</sub> K18E  
217 mutant (AAA to GAA codon change) to generate pRL2-ACP<sub>L</sub>-GFP-mDHFR and pRL2-  
218 ACP<sub>L</sub>(K18E)-GFP-mDHFR for expression of GFP-mDHFR fusions from the mitochondrial  
219 ribosomal protein L2 promoter (37).

220

## 221 **Parasite transfection**

222 Transfections into Dd2<sup>attB</sup> parasites were performed using a variation of the spontaneous uptake  
223 method (38, 39). Briefly, 50 µg each of pINT (29) and the desired pRL2 plasmid were ethanol  
224 precipitated and resuspended in a 0.2 cm electroporation cuvette in 100 µL TE buffer, 100 µL  
225 RPMI 1640 containing 10 mM HEPES-NaOH, pH 7.4, and 200 µL packed uninfected

226 erythrocytes. Erythrocytes were pulsed with 8 square wave pulses of 365 V x 1 ms separated by  
227 0.1 s and were allowed to reseal for 1 hour in a 37°C water bath before allowing parasites to  
228 invade. Drug selection with 2.5 µg/mL Blasticidin S (Research Products International) was  
229 initiated 4 days after transfection.

230

### 231 **Microscopy**

232 For live imaging, parasites were settled onto glass-bottomed microwell dishes (MatTek P35G-  
233 1.5-14-C) in PBS containing 0.4% glucose and 2 µg/mL Hoechst 33342 stain (ThermoFisher  
234 H3570).

235 For fixed imaging, parasites were processed as previously described (40) with  
236 modifications. Briefly, parasites were washed in PBS and fixed in 4% paraformaldehyde  
237 (Electron Microscopy Sciences 15710) and 0.0075% glutaraldehyde (Electron Microscopy  
238 Sciences 16019) in PBS for 20 minutes. Cells were washed once in PBS, resuspended in PBS,  
239 and allowed to settle onto poly-L-lysine-coated coverslips (Corning) for 1 hour. Coverslips were  
240 washed once with PBS, permeabilized in 0.1% Triton X-100 in PBS for 10 minutes, and washed  
241 twice more in PBS. Coverslips were treated with 0.1 mg/mL sodium borohydride in PBS for 10  
242 minutes, washed once in PBS, and blocked in 5% BSA in PBS. Following blocking, parasites  
243 were stained with 1:500 rabbit- $\alpha$ -PfACP (41) diluted in 5% BSA in PBS overnight at 4°C.  
244 Coverslips were washed three times in PBS, incubated for 1 hour in 1:3000 donkey- $\alpha$ -rabbit 568  
245 secondary antibody (ThermoFisher A10042) in 5% BSA in PBS, washed three times in PBS,  
246 mounted onto slides with ProLong Gold antifade reagent with DAPI (ThermoFisher P36935),  
247 and sealed with nail polish prior to imaging.

248 Cells were imaged with a 100X, 1.35 NA objective on an Olympus IX70 microscope  
249 with a DeltaVision system (Applied Precision) controlled with SoftWorx version 4.1.0 and  
250 equipped with a CoolSnap-HQ CCD camera (Photometrics). Brightness and contrast were  
251 adjusted in Fiji (ImageJ) for display purposes.

252

### 253 **Parasite growth assays**

254 For dose-response assays, sorbitol-synchronized ring-stage parasites were grown in 96-well  
255 plates containing 2-fold serial dilutions of WR99210 (Jacobus Pharmaceutical Company). After  
256 3 days of growth, parasites were fixed in 1% paraformaldehyde in PBS and were stained with 50  
257 nM YOYO-1 Iodide (ThermoFisher Y3601). Parasitemia was analyzed on a BD Accuri C6 flow  
258 cytometer. Each biological replicate of dose-response assays was performed in technical  
259 triplicate.

260 For time course growth experiments, sorbitol-synchronized parasites were untreated or  
261 were grown with 10 nM WR99210 with or without 200  $\mu$ M IPP (Isoprenoids, LLC) for 5 days.  
262 Cultures were treated identically in terms of media changes and splitting into fresh erythrocytes.  
263 Samples to assess growth were collected daily, fixed in 1% paraformaldehyde in PBS, and stored  
264 at 4°C until completion of the experiment. Samples were then stained with YOYO-1 and  
265 analyzed as above.

266

### 267 **qPCR**

268 Samples for DNA isolation were harvested daily during growth time course experiments.  
269 Parasites were released from erythrocytes by treatment with 0.1% saponin, washed in PBS, and  
270 stored at -80°C until analysis. Total parasite DNA was isolated using the DNeasy Blood &

271 Tissue kit (Qiagen). qPCR was performed using Power SYBR Green PCR Master Mix (Thermo  
272 Fisher) with 0.15  $\mu$ M each CHT1 F and CHT1 R primers targeting the nuclear gene chitinase or  
273 TufA F and TufA R primers targeting the apicoplast gene elongation factor Tu (30). qPCR was  
274 performed on Applied Biosystems 7900HT or ViiA 7 Real-Time PCR systems with the  
275 following thermocycling conditions: initial denaturation 95°C/10 minutes; 35 cycles of 95°C/1  
276 minute, 56°C/1 minute, 65°C/1 minute; final extension 65°C/10 minutes. Relative quantification  
277 was performed using the  $\Delta\Delta C_t$  method.

278

### 279 **Western blotting**

280 Sorbitol-synchronized parasites were untreated or were treated with 10 nM WR99210 and 200  
281  $\mu$ M IPP for 1 or 3 days. Parasites were separated from RBCs by lysis in 0.1% saponin, washed in  
282 PBS, and were stored at -80°C until analysis. Parasite pellets were resuspended in PBS  
283 containing 1X NuPAGE LDS sample buffer with 50 mM DTT and were heated to 95°C for 10  
284 minutes before separation on NuPAGE Bis-Tris gels and transfer to nitrocellulose. Membranes  
285 were blocked in 0.1% Hammarsten casein (Affymetrix) in 0.2X PBS with 0.01% sodium azide.  
286 Antibody incubations were performed in a 1:1 mixture of blocking buffer and TBST (Tris-  
287 buffered saline with Tween 20: 10 mM Tris, pH 8.0, 150 mM NaCl, 0.25 mM EDTA, 0.05%  
288 Tween 20. Blots were incubated with primary antibody at 4°C overnight at the following  
289 dilutions: 1:20,000 mouse- $\alpha$ -GFP JL-8 (Clontech 632381); 1:4000 rabbit- $\alpha$ -PfClpP (42);  
290 1:20,000 rabbit- $\alpha$ -PfAldolase (Abcam ab207494). Blots were washed once in TBST and were  
291 incubated for 1 hour at room temperature in 1:10,000 dilutions of IRDye 800CW donkey- $\alpha$ -  
292 rabbit or IRDye 680LT goat- $\alpha$ -mouse secondary antibodies (LI-COR Biosciences). Blots were  
293 washed three times in TBST and once in PBS before imaging on a LI-COR Odyssey imager.

294 Band intensities of precursor and mature protein were quantified using Image Studio Lite version  
295 5.2 (LI-COR).

296

## 297 **Statistics**

298 One-way ANOVAs with Tukey's multiple comparisons tests were performed in GraphPad Prism  
299 version 7.04.

300

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309

## 310 **References**

- 311 1. World Health Organization. 2017. World Malaria Report 2017. World Health  
312 Organization, Geneva, Switzerland.
- 313 2. Dondorp AM, Nosten F, Yi P, Das D, Phyto AP, Tarning J, Lwin KM, Ariey F,  
314 Hanpithakpong W, Lee SJ, Ringwald P, Silamut K, Imwong M, Chotivanich K, Lim P,  
315 Herdman T, An SS, Yeung S, Singhasivanon P, Day NP, Lindegardh N, Socheat D,  
316 White NJ. 2009. Artemisinin resistance in *Plasmodium falciparum* malaria. N Engl J  
317 Med 361:455-67.
- 318 3. Ashley EA, Dhorda M, Fairhurst RM, Amaratunga C, Lim P, Suon S, Sreng S, Anderson  
319 JM, Mao S, Sam B, Sopha C, Chuor CM, Nguon C, Sovannaroeth S, Pukrittayakamee S,  
320 Jittamala P, Chotivanich K, Chutasmit K, Suchatsoonthorn C, Runcharoen R, Hien TT,  
321 Thuy-Nhien NT, Thanh NV, Phu NH, Htut Y, Han KT, Aye KH, Mokuolu OA,  
322 Olaosebikan RR, Folaranmi OO, Mayxay M, Khanthavong M, Hongvanthong B, Newton

- 323 PN, Onyamboko MA, Fanello CI, Tshefu AK, Mishra N, Valecha N, Phyo AP, Nosten F,  
324 Yi P, Tripura R, Borrmann S, Bashraheil M, Peshu J, Faiz MA, Ghose A, Hossain MA,  
325 Samad R, Rahman MR, Hasan MM, Islam A, Miotto O, Amato R, MacInnis B, Stalker J,  
326 Kwiatkowski DP, Bozdech Z, Jeeyapant A, Cheah PY, Sakulthaew T, Chalk J, Intharabut  
327 B, Silamut K, Lee SJ, Vihokhern B, Kunasol C, Imwong M, Tarning J, Taylor WJ,  
328 Yeung S, Woodrow CJ, Flegg JA, Das D, Smith J, Venkatesan M, Plowe CV,  
329 Stepniewska K, Guerin PJ, Dondorp AM, Day NP, White NJ, Tracking Resistance to  
330 Artemisinin Collaboration (TRAC). 2014. Spread of artemisinin resistance in  
331 *Plasmodium falciparum* malaria. *N Engl J Med* 371:411-23.
- 332 4. McFadden GI, Reith ME, Munholland J, Lang-Unnasch N. 1996. Plastid in human  
333 parasites. *Nature* 381:482.
- 334 5. Kohler S, Delwiche CF, Denny PW, Tilney LG, Webster P, Wilson RJ, Palmer JD, Roos  
335 DS. 1997. A plastid of probable green algal origin in Apicomplexan parasites. *Science*  
336 275:1485-9.
- 337 6. Sheiner L, Vaidya AB, McFadden GI. 2013. The metabolic roles of the endosymbiotic  
338 organelles of *Toxoplasma* and *Plasmodium* spp. *Curr Opin Microbiol* 16:452-8.
- 339 7. van Dooren GG, Striepen B. 2013. The algal past and parasite present of the apicoplast.  
340 *Annu Rev Microbiol* 67:271-89.
- 341 8. Spork S, Hiss JA, Mandel K, Sommer M, Kooij TW, Chu T, Schneider G, Maier UG,  
342 Przyborski JM. 2009. An unusual ERAD-like complex is targeted to the apicoplast of  
343 *Plasmodium falciparum*. *Eukaryot Cell* 8:1134-45.
- 344 9. Kalanon M, Tonkin CJ, McFadden GI. 2009. Characterization of two putative protein  
345 translocation components in the apicoplast of *Plasmodium falciparum*. *Eukaryot Cell*  
346 8:1146-54.
- 347 10. Agrawal S, van Dooren GG, Beatty WL, Striepen B. 2009. Genetic evidence that an  
348 endosymbiont-derived endoplasmic reticulum-associated protein degradation (ERAD)  
349 system functions in import of apicoplast proteins. *J Biol Chem* 284:33683-91.
- 350 11. Agrawal S, Chung DW, Pons N, van Dooren GG, Prudhomme J, Brooks CF, Rodrigues  
351 EM, Tan JC, Ferdig MT, Striepen B, Le Roch KG. 2013. An apicoplast localized  
352 ubiquitylation system is required for the import of nuclear-encoded plastid proteins.  
353 *PLOS Pathog* 9:e1003426.
- 354 12. Fellows JD, Cipriano MJ, Agrawal S, Striepen B. 2017. A plastid protein that evolved  
355 from ubiquitin and is required for apicoplast protein import in *Toxoplasma gondii*. *mBio*  
356 8:e00950-17.
- 357 13. van Dooren GG, Tomova C, Agrawal S, Humbel BM, Striepen B. 2008. *Toxoplasma*  
358 *gondii* Tic20 is essential for apicoplast protein import. *Proc Natl Acad Sci U S A*  
359 105:13574-9.
- 360 14. Glaser S, van Dooren GG, Agrawal S, Brooks CF, McFadden GI, Striepen B, Higgins  
361 MK. 2012. Tic22 is an essential chaperone required for protein import into the apicoplast.  
362 *J Biol Chem* 287:39505-12.
- 363 15. Sheiner L, Fellows JD, Ovciarikova J, Brooks CF, Agrawal S, Holmes ZC, Bietz I,  
364 Flinner N, Heiny S, Mirus O, Przyborski JM, Striepen B. 2015. *Toxoplasma gondii*  
365 Toc75 functions in import of stromal but not peripheral apicoplast proteins. *Traffic*  
366 16:1254-69.
- 367 16. Goodman CD, Su V, McFadden GI. 2007. The effects of anti-bacterials on the malaria  
368 parasite *Plasmodium falciparum*. *Mol Biochem Parasitol* 152:181-91.



- 369 17. Dahl EL, Rosenthal PJ. 2007. Multiple antibiotics exert delayed effects against the  
370 *Plasmodium falciparum* apicoplast. Antimicrob Agents Chemother 51:3485-90.
- 371 18. de Koning-Ward TF, Gilson PR, Crabb BS. 2015. Advances in molecular genetic systems  
372 in malaria. Nat Rev Microbiol 13:373-87.
- 373 19. Rottmann M, McNamara C, Yeung BK, Lee MC, Zou B, Russell B, Seitz P, Plouffe DM,  
374 Dharia NV, Tan J, Cohen SB, Spencer KR, Gonzalez-Paez GE, Lakshminarayana SB,  
375 Goh A, Suwanarusk R, Jegla T, Schmitt EK, Beck HP, Brun R, Nosten F, Renia L,  
376 Dartois V, Keller TH, Fidock DA, Winzeler EA, Diagana TT. 2010. Spiroindolones, a  
377 potent compound class for the treatment of malaria. Science 329:1175-80.
- 378 20. Ganesan SM, Falla A, Goldfless SJ, Nasamu AS, Niles JC. 2016. Synthetic RNA-protein  
379 modules integrated with native translation mechanisms to control gene expression in  
380 malaria parasites. Nat Commun 7:10727.
- 381 21. Amberg-Johnson K, Hari SB, Ganesan SM, Lorenzi HA, Sauer RT, Niles JC, Yeh E.  
382 2017. Small molecule inhibition of apicomplexan FtsH1 disrupts plastid biogenesis in  
383 human pathogens. Elife 6:e29865.
- 384 22. Armstrong CM, Goldberg DE. 2007. An FKBP destabilization domain modulates protein  
385 levels in *Plasmodium falciparum*. Nat Methods 4:1007-9.
- 386 23. Muralidharan V, Oksman A, Iwamoto M, Wandless TJ, Goldberg DE. 2011. Asparagine  
387 repeat function in a *Plasmodium falciparum* protein assessed via a regulatable fluorescent  
388 affinity tag. Proc Natl Acad Sci U S A 108:4411-6.
- 389 24. Eilers M, Schatz G. 1986. Binding of a specific ligand inhibits import of a purified  
390 precursor protein into mitochondria. Nature 322:228-32.
- 391 25. Gehde N, Hinrichs C, Montilla I, Charpian S, Lingelbach K, Przyborski JM. 2009.  
392 Protein unfolding is an essential requirement for transport across the parasitophorous  
393 vacuolar membrane of *Plasmodium falciparum*. Mol Microbiol 71:613-28.
- 394 26. Gruring C, Heiber A, Kruse F, Flemming S, Franci G, Colombo SF, Fasana E, Schoeler  
395 H, Borgese N, Stunnenberg HG, Przyborski JM, Gilberger TW, Spielmann T. 2012.  
396 Uncovering common principles in protein export of malaria parasites. Cell Host Microbe  
397 12:717-29.
- 398 27. Mesen-Ramirez P, Reinsch F, Blancke Soares A, Bergmann B, Ullrich AK, Tenzer S,  
399 Spielmann T. 2016. Stable translocation intermediates jam global protein export in  
400 *Plasmodium falciparum* parasites and link the PTEX component EXP2 with translocation  
401 activity. PLOS Pathog 12:e1005618.
- 402 28. Foth BJ, Ralph SA, Tonkin CJ, Struck NS, Fraunholz M, Roos DS, Cowman AF,  
403 McFadden GI. 2003. Dissecting apicoplast targeting in the malaria parasite *Plasmodium*  
404 *falciparum*. Science 299:705-8.
- 405 29. Nkrumah LJ, Muhle RA, Moura PA, Ghosh P, Hatfull GF, Jacobs WR, Jr., Fidock DA.  
406 2006. Efficient site-specific integration in *Plasmodium falciparum* chromosomes  
407 mediated by mycobacteriophage Bxb1 integrase. Nat Methods 3:615-21.
- 408 30. Yeh E, DeRisi JL. 2011. Chemical rescue of malaria parasites lacking an apicoplast  
409 defines organelle function in blood-stage *Plasmodium falciparum*. PLOS Biol  
410 9:e1001138.
- 411 31. Chou TF, Brown SJ, Minond D, Nordin BE, Li K, Jones AC, Chase P, Porubsky PR,  
412 Stoltz BM, Schoenen FJ, Patricelli MP, Hodder P, Rosen H, Deshaies RJ. 2011.  
413 Reversible inhibitor of p97, DBeQ, impairs both ubiquitin-dependent and autophagic  
414 protein clearance pathways. Proc Natl Acad Sci U S A 108:4834-9.



- 415 32. Chou TF, Li K, Frankowski KJ, Schoenen FJ, Deshaies RJ. 2013. Structure-activity  
416 relationship study reveals ML240 and ML241 as potent and selective inhibitors of p97  
417 ATPase. *ChemMedChem* 8:297-312.
- 418 33. Magnaghi P, D'Alessio R, Valsasina B, Avanzi N, Rizzi S, Asa D, Gasparri F, Cozzi L,  
419 Cucchi U, Orrenius C, Polucci P, Ballinari D, Perrera C, Leone A, Cervi G, Casale E,  
420 Xiao Y, Wong C, Anderson DJ, Galvani A, Donati D, O'Brien T, Jackson PK, Isacchi A.  
421 2013. Covalent and allosteric inhibitors of the ATPase VCP/p97 induce cancer cell death.  
422 *Nat Chem Biol* 9:548-56.
- 423 34. Anderson DJ, Le Moigne R, Djakovic S, Kumar B, Rice J, Wong S, Wang J, Yao B,  
424 Valle E, Kiss von Soly S, Madriaga A, Soriano F, Menon MK, Wu ZY, Kampmann M,  
425 Chen Y, Weissman JS, Aftab BT, Yakes FM, Shawver L, Zhou HJ, Wustrow D, Rolfe  
426 M. 2015. Targeting the AAA ATPase p97 as an approach to treat cancer through  
427 disruption of protein homeostasis. *Cancer Cell* 28:653-665.
- 428 35. Hyer ML, Milhollen MA, Ciavarrri J, Fleming P, Traore T, Sappal D, Huck J, Shi J,  
429 Gavin J, Brownell J, Yang Y, Stringer B, Griffin R, Bruzzese F, Soucy T, Duffy J,  
430 Rabino C, Riceberg J, Hoar K, Lublinsky A, Menon S, Sintchak M, Bump N, Pulukuri  
431 SM, Langston S, Tirrell S, Kuranda M, Veiby P, Newcomb J, Li P, Wu JT, Powe J, Dick  
432 LR, Greenspan P, Galvin K, Manfredi M, Claiborne C, Amidon BS, Bence NF. 2018. A  
433 small-molecule inhibitor of the ubiquitin activating enzyme for cancer treatment. *Nat*  
434 *Med* 24:186-193.
- 435 36. Boucher MJ, Ghosh S, Zhang L, Lal A, Jang SW, Ju A, Zhang S, Wang X, Ralph SA,  
436 Zou J, Elias JE, Yeh E. 2018. Integrative proteomics and bioinformatic prediction enable  
437 a high-confidence apicoplast proteome in malaria parasites. *PLOS Biol* 16:e2005895.
- 438 37. Balabaskaran Nina P, Morrissey JM, Ganesan SM, Ke H, Pershing AM, Mather MW,  
439 Vaidya AB. 2011. ATP synthase complex of *Plasmodium falciparum*: dimeric assembly  
440 in mitochondrial membranes and resistance to genetic disruption. *J Biol Chem*  
441 286:41312-22.
- 442 38. Deitsch K, Driskill C, Wellems T. 2001. Transformation of malaria parasites by the  
443 spontaneous uptake and expression of DNA from human erythrocytes. *Nucleic Acids Res*  
444 29:850-3.
- 445 39. Wagner JC, Platt RJ, Goldfless SJ, Zhang F, Niles JC. 2014. Efficient CRISPR-Cas9-  
446 mediated genome editing in *Plasmodium falciparum*. *Nat Methods* 11:915-8.
- 447 40. Tonkin CJ, van Dooren GG, Spurck TP, Struck NS, Good RT, Handman E, Cowman AF,  
448 McFadden GI. 2004. Localization of organellar proteins in *Plasmodium falciparum* using  
449 a novel set of transfection vectors and a new immunofluorescence fixation method. *Mol*  
450 *Biochem Parasitol* 137:13-21.
- 451 41. Gallagher JR, Prigge ST. 2010. *Plasmodium falciparum* acyl carrier protein crystal  
452 structures in disulfide-linked and reduced states and their prevalence during blood stage  
453 growth. *Proteins* 78:575-88.
- 454 42. El Bakkouri M, Pow A, Mulichak A, Cheung KL, Artz JD, Amani M, Fell S, de Koning-  
455 Ward TF, Goodman CD, McFadden GI, Ortega J, Hui R, Houry WA. 2010. The Clp  
456 chaperones and proteases of the human malaria parasite *Plasmodium falciparum*. *J Mol*  
457 *Biol* 404:456-77.
- 458

459 **Tables**

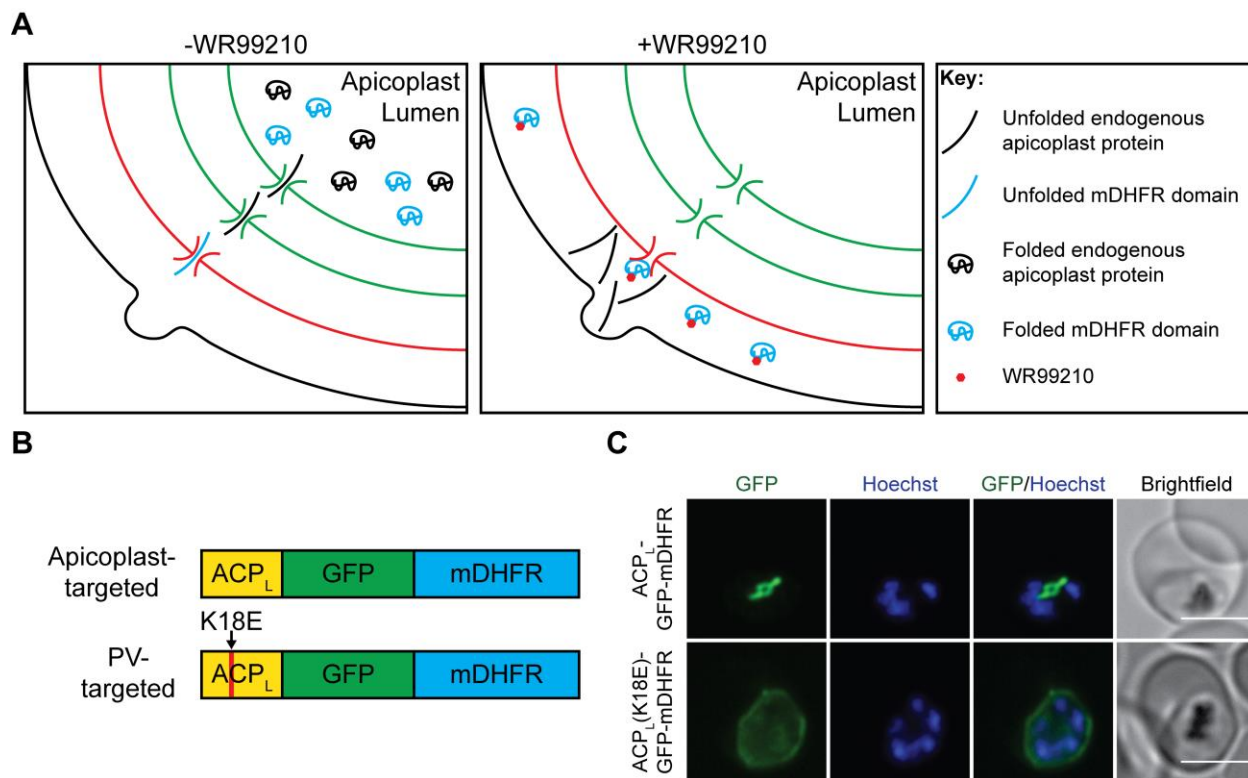
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**Table 1. Primers used in this study.**

<b>Name</b>	<b>Sequence</b>	<b>Description</b>
MB119	TAAAAACCCACGTACGATGAGTAAAGG AGAAGAACTTTTC	Amplification of GFP to generate GFP-mDHFR fusion
MB120	TCGAACCATGGTACCTTTGTATAGTTCA TCCATGCC	Amplification of GFP to generate GFP-mDHFR fusion
MB121	GGTACCATGGTTCGACCATTGAACTGC	Amplification of mDHFR to generate GFP-mDHFR fusion
MB122	ATAACTCGACCTTAAGTTAGTCTTTCTTC TCGTAGACTTCAAACCTTATAC	Amplification of mDHFR to generate GFP-mDHFR fusion
TufA F	GATATTGATTCAGCTCCAGAAGAAA	Apicoplast genome qPCR; from Yeh and DeRisi <i>PLoS Biol</i> 2011
TufA R	ATATCCATTTGTGTGGCTCCTATAA	Apicoplast genome qPCR; from Yeh and DeRisi <i>PLoS Biol</i> 2011
CHT1 F	TGTTTCCTTCAACCCCTTTT	Nuclear genome qPCR; from Yeh and DeRisi <i>PLoS Biol</i> 2011
CHT1 R	TAATCCAAACCCGTCTGCTC	Nuclear genome qPCR; from Yeh and DeRisi <i>PLoS Biol</i> 2011

461

462 **Figures and Legends**



463

464 **Figure 1. Generation of a protein-level conditional tool to disrupt apicoplast protein**

465 **import.** (A) Model for protein-level disruption of apicoplast protein import by a conditional

466 stabilization domain. In the absence of WR99210, an apicoplast-targeted mDHFR domain

467 successfully targets to the apicoplast lumen along with endogenous apicoplast proteins. Addition

468 of WR99210 stabilizes the mDHFR domain to prevent unfolding, which causes the domain to

469 stall during translocation across one or multiple apicoplast membranes and block import of

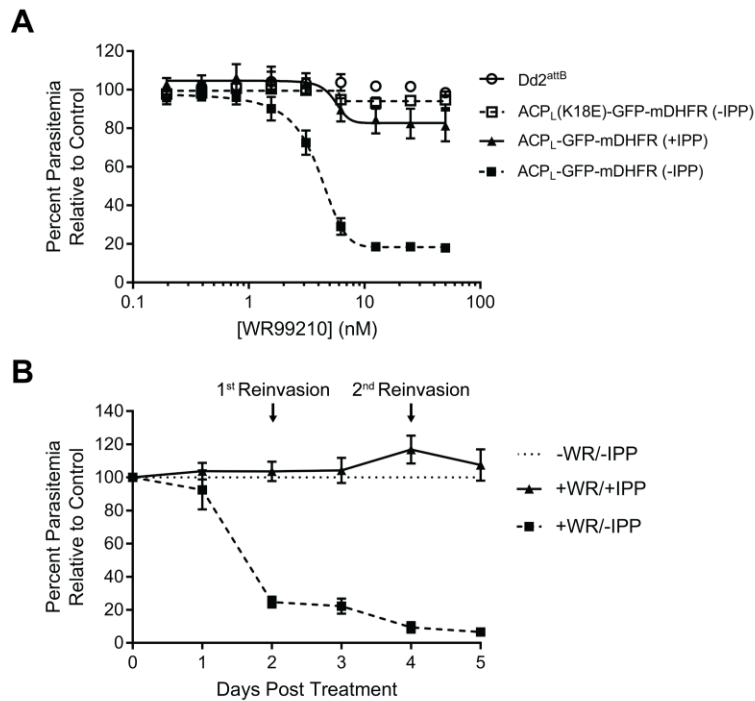
470 essential endogenous apicoplast cargo. (B) Schematic (not to scale) of constructs targeting GFP-

471 mDHFR to the apicoplast via the ACP leader sequence or to the PV via a mutant ACP leader

472 sequence. (C) Live-cell imaging of GFP-mDHFR fusions. Nuclei were stained with Hoechst.

473 Brightness/contrast adjustments were not held constant between the two cell lines due to

474 differences in GFP fluorescence intensity in the apicoplast versus the PV. Scale bars, 5 μm.



475

476 **Figure 2. Conditional stabilization of apicoplast-targeted GFP-mDHFR causes apicoplast-**

477 **specific growth inhibition in the first lytic cycle.** (A) Growth of parental Dd2<sup>attB</sup>,

478 mDHFR, and ACP<sub>L</sub>(K18E)-GFP-mDHFR parasites after 3 days in response to increasing doses

479 of WR99210. ACP<sub>L</sub>-GFP-mDHFR parasites were assayed in both the presence and absence of

480 200 μM IPP. (B) Growth of ACP<sub>L</sub>-GFP-mDHFR parasites in the presence of 10 nM WR99210

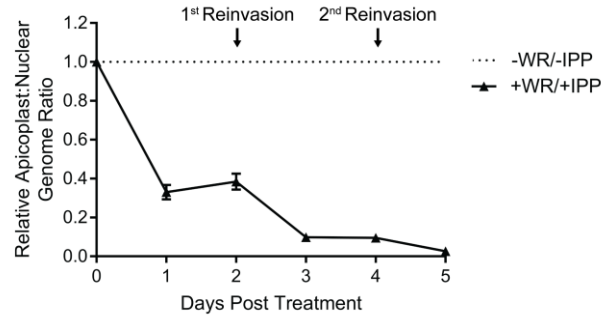
481 over a 5-day time course. Parasites were grown in the presence or absence of 200 μM IPP, and

482 parasitemia was normalized to an untreated control at each time point. Error bars in both panels

483 represent standard deviation of the mean of 3 biological replicates. Biological replicates in (A)

484 were performed in technical triplicate.

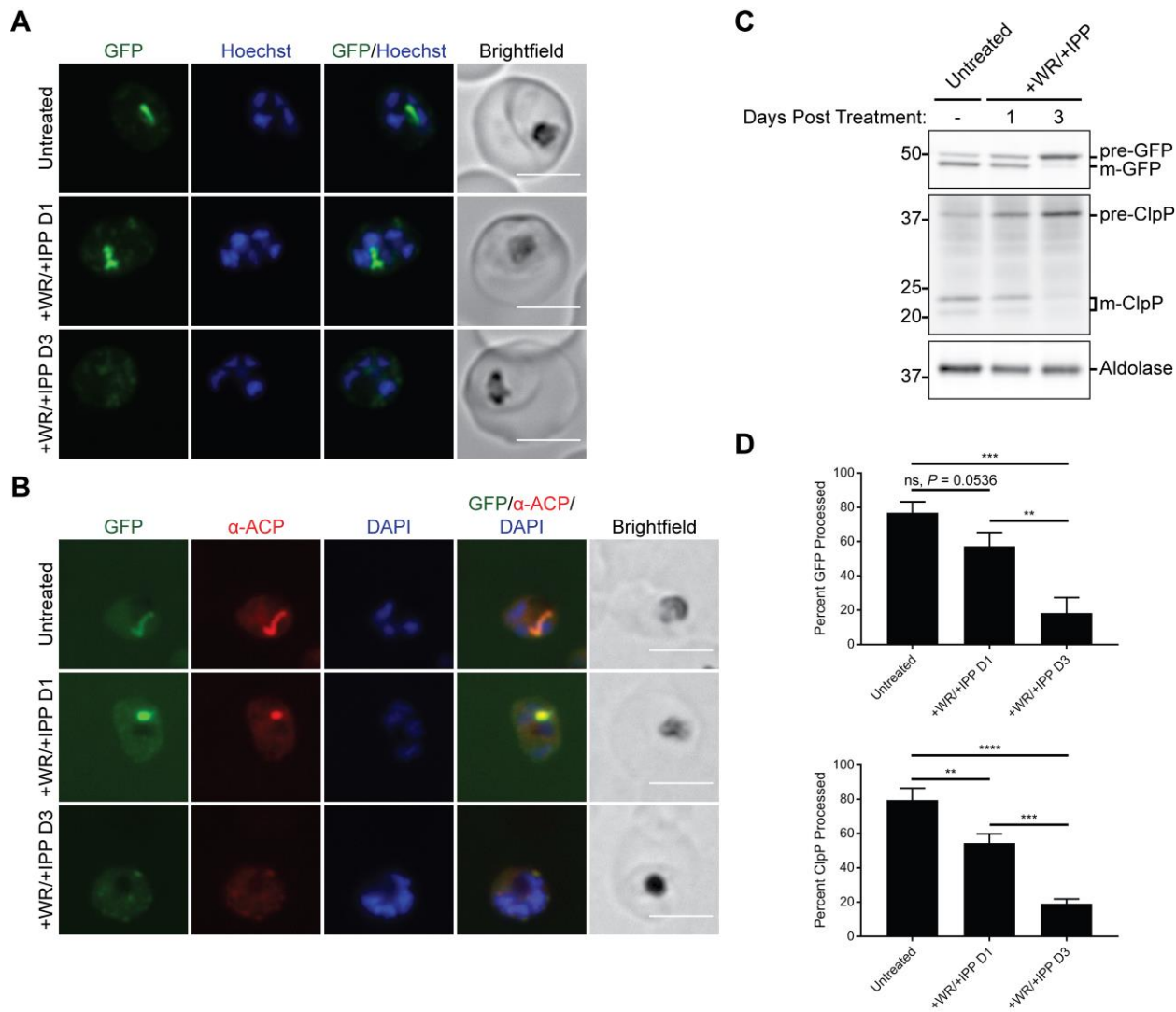
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487 **Figure 3. Conditional stabilization of apicoplast-targeted GFP-mDHFR causes an**  
488 **apicoplast biogenesis defect.** The relative apicoplast:nuclear genome ratio of ACP<sub>L</sub>-GFP-  
489 mDHFR parasites grown in 10 nM WR99210 and 200 μM IPP was measured by qPCR over 5  
490 days of treatment. Values are normalized to an untreated control at each time point. Error bars  
491 represent standard deviation of the mean of 3 biological replicates, each analyzed in technical  
492 triplicate.

493



494

495 **Figure 4. Conditional stabilization of apicoplast-targeted GFP-mDHFR disrupts import of**

496 **endogenous apicoplast cargo across the apicoplast membranes. ACP<sub>L</sub>-GFP-mDHFR**

497 parasites were grown with 10 nM WR99210 and 200 μM IPP for either 1 or 3 days

498 (corresponding to the same lytic cycle or the first lytic cycle post-treatment, respectively). (A)

499 Live imaging of Hoechst-stained parasites. Scale bars, 5 μm. (B) Fixed imaging of parasites

500 stained with an antibody against the endogenous apicoplast marker ACP (recognizing an epitope

501 not present on the ACP<sub>L</sub> fused to GFP-mDHFR). Scale bars, 5 μm. (C) Western blot to assess

502 transit peptide processing of ACP<sub>L</sub>-GFP-mDHFR and the endogenous apicoplast protein ClpP.

503 pre-, precursor (unprocessed) protein; m-, mature (processed) protein. (D) Quantification of  
504 transit peptide processing for ACP<sub>L</sub>-GFP-mDHFR and ClpP. Data are expressed as the  
505 percentage of total GFP or ClpP signal that is mature (processed). Error bars represent standard  
506 deviation of the mean of 3 biological replicates. \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$ , one-  
507 way ANOVA with Tukey's multiple comparisons test. ns, not significant.  
508  
509