

Figure S1. Variability in the C-terminal sequence of HCF101 homologs. Alignment of the C-terminal region of HCF101 homologs from different organisms, including the plant *Arabidopsis thaliana*, the two isoforms of Apicomplexa-relative photosynthetic algae *Chromera velia*, as well as several Apicomplexa species (*Toxoplasma gondii*, *Eimeria tenella*, *Plasmodium falciparum* and apicoplast-less *Cryptosporidium parvum*). The tryptophan-containing targeting complex recognition signal is highlighted in yellow. Neighbor-Joining consensus tree was obtained with protein alignment using Jukes-Cantor genetic distance model 100 bootstrap replicates (bootstrap values indicated at the base of the nodes).

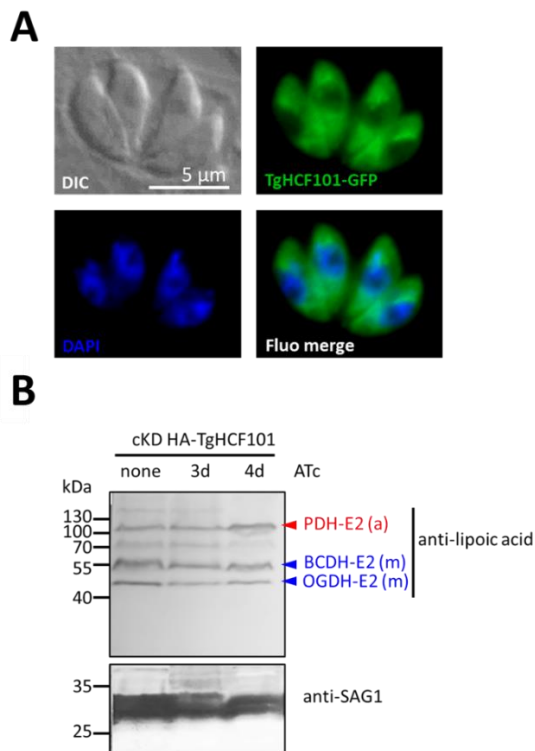


Figure S2. TgHCF101 is not associated with the apicoplast or its metabolism. **A.** Fluorescent imaging of *T. gondii* tachyzoites ectopically-expressing a GFP-fused copy of TgHCF101, DNA was stained with DAPI. Scale bar= 5 μ m. **B.** Immunoblot analysis of the lipoylation profile of *T. gondii* tachyzoites, typically showing apicoplast (Pyruvate dehydrogenase subunit E2, PDH-E2) and mitochondrial (Branched-chain 2-oxo acid dehydrogenase, BCDH-E2 and 2-oxoglutarate dehydrogenase, OGDH-E2) proteins that are largely unaffected upon TgHCF101 depletion by incubation with ATc for up to 4 days. Anti-SAG1 antibody is used as a loading control.

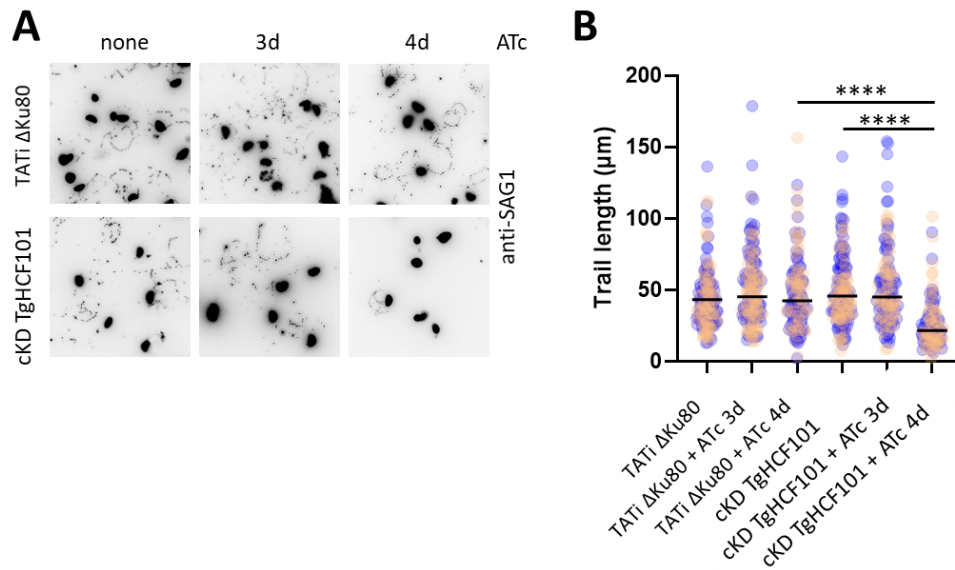


Figure S3. TgHCF101 depletion does not initially impact parasite motility. **A.** Representative images of a gliding motility assay selected out of 3 independent replicates. The trails left behind by gliding parasites were detected using anti-SAG1 antibody. TgHCF101 conditional mutant and parental cell line (TATI Δ Ku80) were grown for 3 or 4 days in the presence of ATc. **B.** Quantification of SAG1 trail length showed in (A) produced by parasites measured on 10 randomly selected fields. At least 100 trails were measured for each dataset, values represented are means of $n=3$ independent biological replicates, **** p -value ≤ 0.0001 , Student's t -test.

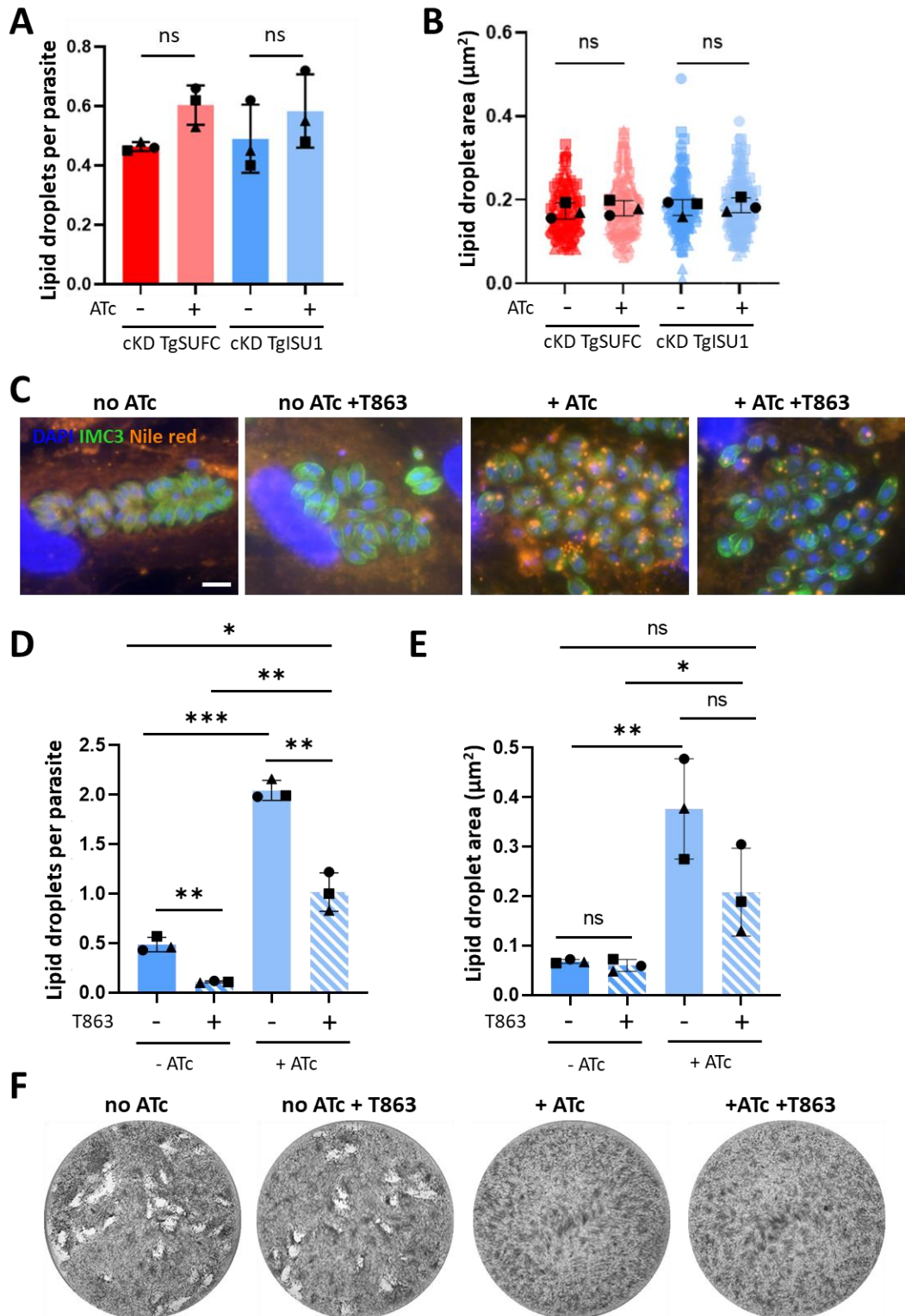


Figure S4. Lipid droplet induction is specific of TgHCF101 depletion and is not responsible for parasite demise. **A.** and **B.** correspond to quantification of the number and area of lipid droplets, respectively, in apicoplast (cKD-TgSUFC) and mitochondrion (cKD-TgISU1) Fe-S cluster synthesis mutants grown in the absence or presence of ATc for up to 72h. 100 parasites were analyzed per condition. Values are the mean \pm SD of $n=3$ independent biological replicates; ns, not significant (p -value ≥ 0.05 , Student's t -test). **C.** Immunofluorescence assay of parasites from the cKD-TgHCF101 cell line treated for 72h with or in the absence of ATc and supplemented or not with diacylglycerol acyltransferase inhibitor T863.

Lipid droplets were detected with Nile red (orange), parasites are outlined with anti-IMC3 antibody (green) and DNA is stained with DAPI. Scale bar= 5 μ m. **D.** and **E.** correspond to the quantification of the number and area of lipid droplet, 100 parasites were analyzed per condition. Parasites were grown in the absence of ATc for the parental (TATi Δ Ku80) and transgenic (cKD HA-TgHCF101) cell lines or for 72h with ATc treatment, supplemented or not with T863. Values are represented as the mean \pm SD of n=3 independent biological replicates; ns, not significant (p-value \geq 0.05), * p-value \leq 0.05, ** p-value \leq 0.01 and *** p-value $<$ 0.001, Student's *t*-test. **F.** Plaque assays were carried out by infecting a monolayer of HFFs with cKD HA-TgHCF101 cell lines for 7 days in the presence or absence of ATc and supplemented or not with T863.

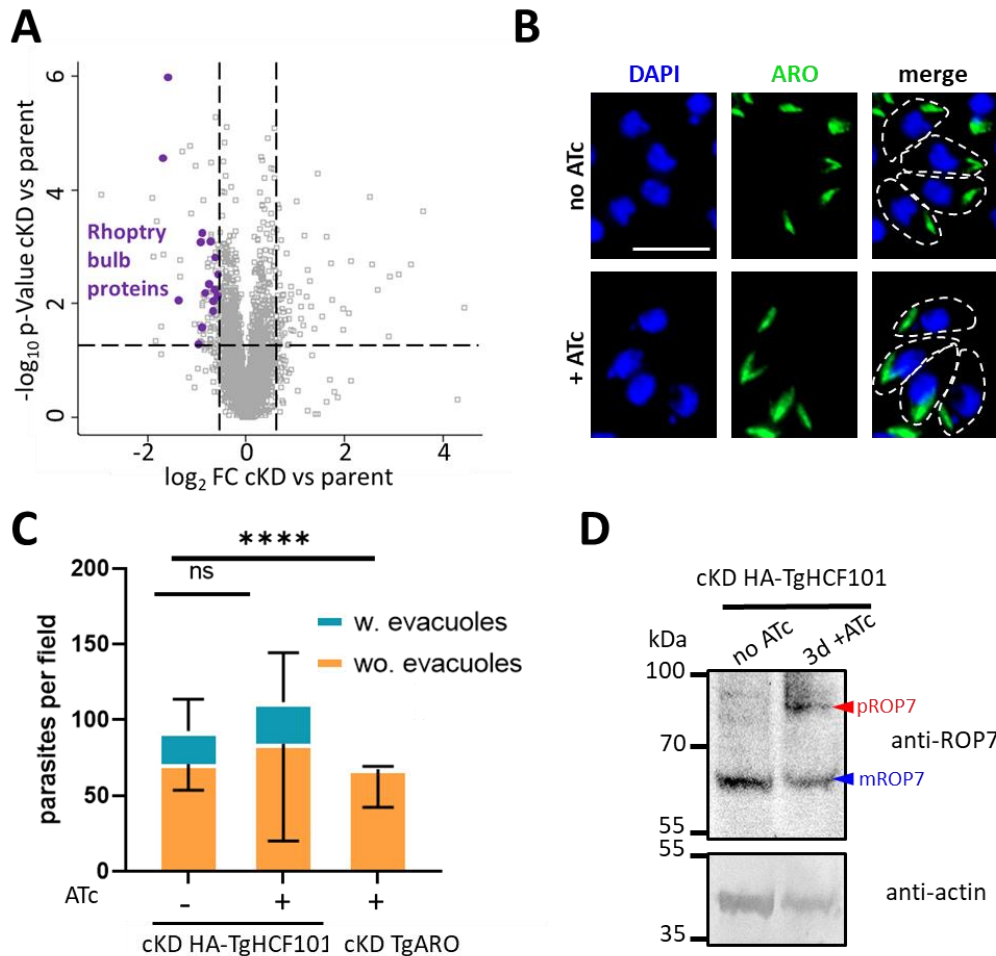


Figure S5. Depletion of TgHCF101 has no significant impact on rhoptries. **A.** Volcano plot showing differential expression of proteins impacted by TgHCF101 depletion after 72h of ATc treatment analyzed by label free quantitative proteomic. X-axis correspond to the \log_2 of the Fold-change, Y-axis correspond to the $-\log_{10}$ of the p-value when comparing cKD-TgHCF101 expression values to the TATi Δ Ku80 parental cell line. Statistical analyses were performed with ANOVA from 4 independent biological replicates. Cut-offs were set at ≤ 1.5 - or ≥ 1.5 -FC and p-value ≤ 0.05 . Significant hits corresponding to rhoptry bulb protein proteins were highlighted in purple on the graph. **B.** Immunofluorescence assay of parasites from the cKD HA-TgHCF101 cell line pre-treated for 48h and allowed to grow on HFF coated coverslips for another 24h with or in the absence of ATc. Rhoptries were detected with anti-ARO antibody (green), parasites are outlined with white dotted lines and DNA is stained with DAPI. Scale bar= 5 μ m. **C.** Quantification of rhoptry secretion events (evacuoles) in the cKD HA-TgHCF101 mutant upon TgHCF101 depletion for 72h. The TgARO conditional knock-down cell line serves as a control of rhoptry secretion defect upon ATc treatment. Parasites with and without evacuoles were counted on 20 randomly selected fields, with 3 technical replicates for each biological replicate. Values are represented as the mean \pm SD of n=3 independent biological replicates; ns, not significant (p-value \geq 0.05), * p-value \leq 0.05, **** a p-value \leq 0.001, Student's t-test. **D.** Immunoblot analysis of the expression of protein TgROP7 upon depletion of TgHCF101 for 3 days. The pro-form of TgROP7 (pROP7) is highlighted by a red arrow and the mature form (mROP7) by a blue arrow. TgROP7 signal was detected by anti-ROP7 antibody and anti-actin antibody was used as a loading control.

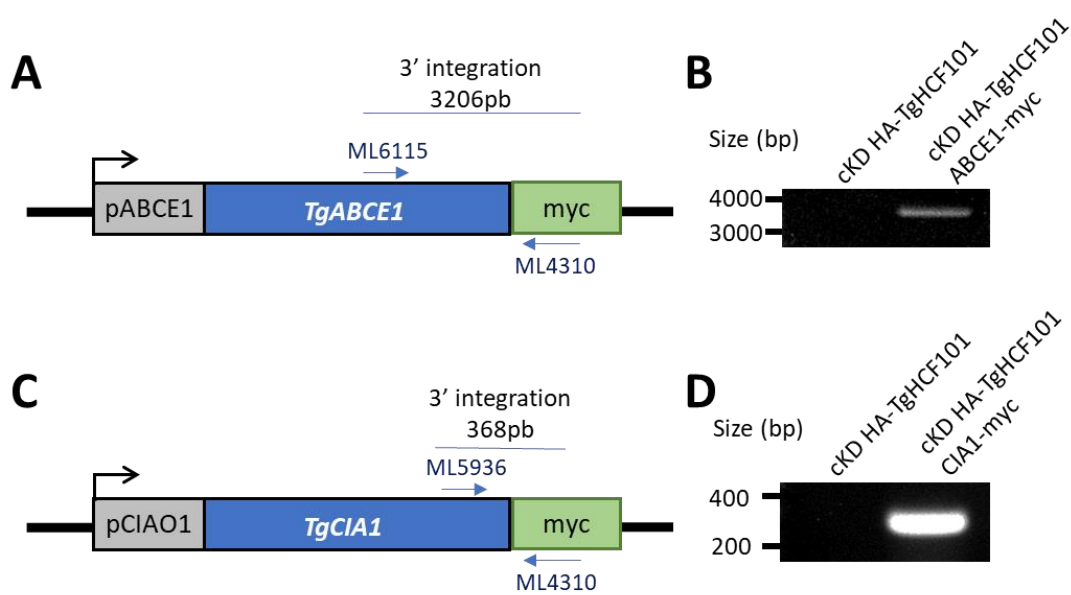


Figure S6. Constructs for tagging TgABCE1 and TgCIA1 in the cKD HA-TgHCF101 mutant background. **A.** and **C.** correspond to schematic representations of the strategy used to add a C-terminal myc tag to proteins TgABCE1 (A) and TgCIA1 (C) by homologous recombination at the native locus. Chloramphenicol was used to select transgenic parasites. **B.** and **D.** correspond to diagnostic PCRs on genomic DNA from parental cell line (cKD HA-TgHCF101) or new clonal cell lines, in order to check for the integration of the sequence coding for the myc tag using primers highlighted on the (A) and (C) schemes.

PF09811 PFAM domain
Essential protein Yae1, N terminal

IPR052436 Interpro domain
LTO1 complex adapter protein

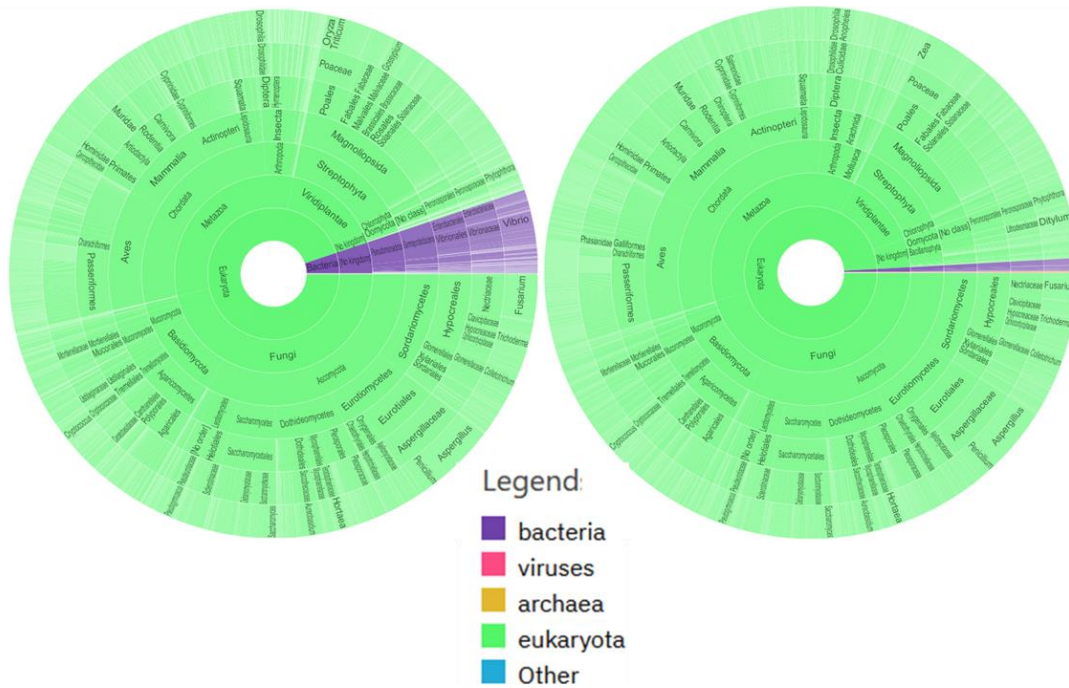


Figure S7. CIA adapters YAE1 and LTO1 are restricted to specific eukaryotic lineages. Sunburst representation of the distribution of YAE1 and LTO1 domains as retrieved in the Interpro database (<https://www.ebi.ac.uk/interpro/>).